Co-Inhibitory Suppression of T Cell Activation by CD40 Protects from Obesity and Adipose Tissue Inflammation in Mice

Running title: Wolf et al.; CD40 and the metabolic syndrome

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Abstract

**Background**—Co-stimulatory cascades such as the CD40L-CD40 dyad enhance immune cell activation and inflammation during atherosclerosis. Here, we tested the hypothesis that CD40 directly modulates traits of the metabolic syndrome in diet-induced obesity (DIO) in mice.

**Methods and Results**—To induce the metabolic syndrome, WT or CD40-/- mice consumed a high fat diet for 20 weeks. Unexpectedly, CD40-/- mice exhibited increased weight gain, impaired insulin secretion, augmented accumulation of inflammatory cells in adipose tissue, and enhanced pro-inflammatory gene expression. This pro-inflammatory and adverse metabolic phenotype could be transplanted into WT mice by reconstitution with CD40-deficient lymphocytes, indicating a major role for CD40 on T- or B cells in this context. Conversely, therapeutic activation of CD40 signaling by the stimulating antibody FGK45 abolished further weight gain during the study, lowered glucose levels, improved insulin sensitivity, and suppressed adipose tissue inflammation. Mechanistically, CD40 activation decreased expression of pro-inflammatory cytokines in T cells, but not in B cells or macrophages. Finally, re-population of lymphocyte-free Rag1-/- mice with CD40-/- T cells provoked dysmetabolism and inflammation, corroborating a protective role of CD40 on T cells in the metabolic syndrome. Finally, levels of sCD40 showed a positive association with obesity in humans, suggesting clinical relevance of our findings.

**Conclusions**—We present the surprising finding that CD40 deficiency on T cells aggravates, while activation of CD40 signaling improves, adipose tissue inflammation and its metabolic complications. Positive modulation of the CD40 pathway might, therefore, describe a novel therapeutic concept against cardio-metabolic disease.

**Key words:** obesity, inflammation, CD40/CD40L, adipose tissue, T-cell
Introduction

The metabolic syndrome comprises a cluster of risk factors including obesity, insulin resistance, hepatic steatosis, and dyslipidemia. It associates with a variety of cardiovascular diseases, such as atherosclerosis, myocardial infarction, and stroke. Chronic, low-grade inflammation in key metabolic organs, such as the liver and visceral adipose tissue (VAT), accompanies this cluster. Considerable evidence implicates the adaptive immune system in the regulation of adipose tissue inflammation and glucose homeostasis. Immune effector cells modulate inflammatory and metabolic cascades throughout all stages of obesity. T cells, B cells, macrophages, and mast cells infiltrate VAT and orchestrate local and systemic inflammatory responses by interacting with each other and with resident adipocytes. CD4+ T-helper (T\textsubscript{H}) cells, CD4+ T-regulatory (T\textsubscript{reg}) cells, CD8+ T cells, and related chemo- and cytokines, such as RANTES and IFN-\gamma, co-localize within the inflammatory cell compartment in adipose tissue. In lean adipose tissue, the vast majority of T lymphocytes share features of anti-inflammatory, IL-13-, IL-4-, and IL-10-secreting T\textsubscript{H2} or T\textsubscript{reg} cells. In obesity, pro-inflammatory T\textsubscript{H1} cells expressing IFN-\gamma overwhelm T\textsubscript{H2} cells. T\textsubscript{H1} cells, in turn, activate pro-inflammatory cytokine-secreting macrophages, and promote their conversion from M2-like, IL-10-secreting, alternatively activated macrophages (AAMs) to classically activated, M1-like macrophages. Despite description of the kinetics of cellular infiltration and the associated cytokine/chemokine profiles during the development of obesity, the underlying cause modulating T cell polarization in adipose tissue inflammation remains poorly understood. Activation and differentiation of T cells requires co-stimulatory signaling. The co-stimulatory molecule CD40, a member of the TNF receptor superfamily, critically regulates B cell and T cell function in adaptive immunity and inflammation by interacting with CD40L (CD154). We and others recently showed that
pre-adipocytes and adipocytes express CD40, and proposed that adipose tissue inflammation requires CD40L action\textsuperscript{15-17}. In contrast, Guo et al. recently demonstrated that mice with a genetic deficiency of the CD40 receptor exhibited a hyper-inflammatory phenotype in a model of diet-induced obesity (DIO), suggesting that CD40 surprisingly plays a protective role in the metabolic syndrome\textsuperscript{18}. In light of CD40’s proposed pro-inflammatory role in various disease models, the findings of Guo et al. raise interesting questions, such as the exact molecular mechanism, the cellular origin of CD40’s phenotype, and whether CD40 might be responsive to therapeutic stimulation. Here, we aim to address these questions and to further investigate and confirm the effects of CD40 on metabolic and inflammatory phenotype.

**Methods**

Expanded methods are available in the Online Supplemental Material

**Animal Protocols**

Male CD40\textsuperscript{-/-} mice (B6.129P2-\textit{Cd40tm1Kik}/J, Jackson, USA) on a C57BL/6J background (approximate) and aged-matched wild-type C57BL/6J (WT) mice consumed high-fat diet (HFD) or low-fat diet (LFD) ad libitum for 20 weeks. As indicated, male WT mice were injected three times per week (100 \textmu g i.p.) with the agonistic anti-CD40 antibody FGK45 (Miltenyi, Germany) or isotype control, and were pair-fed with HFD for 6 weeks. After bone marrow transplantations or adoptive transfers of T cells, animals were pair-fed with HFD as indicated. All experimental protocols were approved by the animal ethics committee of the Alfred Medical Research and Education Precinct (AMREP), Melbourne, Australia and the animal ethics committee at the University of Freiburg, Germany. All procedures were carried out in accordance with institutional guidelines.
Bone marrow transplantation

Recipient mice were lethally irradiated with one dose of 700cGy (Gammacell Exactor 40). Bone marrow of donor animals was filtered, washed, and injected at a concentration of 1x10^7 cells into the tail vein.

Adoptive transfer of T cells

Splenic T cells from WT or CD40^-/- mice were isolated by negative cell separation (Stem Cell Technologies, USA). Purity was >95% in flow cytometry. 5x10^6 cells were transferred into Rag-1^-/- mice by intraperitoneal injection. Donor animals subsequently consumed HFD for 8 weeks.

Body composition analysis

Body composition was assessed by EchoMRI (Echo Medical Systems, USA).

Metabolic caging

Animals were allowed to acclimatize to metabolic cages (Oxymax/CLAMS, Columbus Instruments, USA) prior to the observation period. Food intake, ambulatory movement, heat production, O_2 consumption, and CO_2 production were assessed and recorded during a 24-hour observation period.

Intraperitoneal glucose tolerance testing (GTT) and insulin tolerance testing (ITT)

Mice were starved 6 hours prior to the procedures, and received intraperitoneal glucose injections (0.25-1g/kg lean body mass) for GTT. For ITT, mice were injected intraperitoneally with insulin (0.1-0.5U/kg).

Preparation of the stromal vascular fraction (SVF)

Murine epididymidal fat pads were minced and digested with collagenase B (0.5mg/ml). Digested tissue was filtered through nylon mesh (100μm). SVF and adipocyte fraction were obtained from the resulting pellet and supernatant, respectively.
Flow cytometry

Cells were washed in PBS, Fc-Receptors were blocked by anti-CD16/CD32, and cells were labeled with the indicated antibodies (all from eBioscience, USA) before quantification with a flow cytometer (BD FACS Canto II, BD, USA). Leukocyte populations were identified upon cell surface expression of the indicated antigens: T-helper cells (CD3+CD4+CD8⁻), cytotoxic T cells (CD3⁺CD4⁺CD8⁺), T-regulatory cells (CD4⁺CD25⁺FoxP3⁺), B cells (CD19⁺), M1-macrophages (F4/80⁺CD11c⁺), M2-macrophages (F4/80⁺CD11c⁻), dendritic cells (CD11c⁺), inflammatory monocytes (CD11b⁺CD115⁺Gr-1⁺), non-inflammatory monocytes (CD11b⁺CD115⁺Gr-1⁻), and endothelial cells (VCAM-1⁺).

Isolation of total RNA and quantitative real-time PCR

Isolation of RNA and qPCR was performed as previously described. Alternatively, RNA samples were screened using a MouseWG-6 v2 Expression BeadChip (Illumina, USA).

Quantification of CD40 expression

The stromal vascular fraction (SVF) and the adipocyte fraction were separated and total protein was isolated. Equal concentrations of protein were either loaded on Western blot, stained with a specific anti-CD40 antibody and visualized, or CD40 concentrations were quantified by ELISA (R&D, USA).

Histology and immunohistochemistry (IHC)

Adipose tissue was fixed, dehydrated, embedded in paraffin, and cut into 6-μm sections. For IHC, sections were blocked with 4% rabbit serum, incubated for 1.5 hours at RT with anti-CD8 (Abcam, USA). Livers were embedded in Tissue-Tek compound (Sakura Finetek, Japan) and incubated in Oil-Red-O staining solution.

Quantification of intracellular cytokines
Intracellular proteins were quantified by flow cytometry in splenocytes or cells of the stromal-vascular fraction. Cells were re-stimulated by phorbol-myristate-acetate (PMA, 81nM), ionomycin (1.34μM), brefeldin A (10μM) in RPMI for 15 hours, or by cultivation on mouse anti-CD3 coated cell culture plates for the indicated time.

**Measurement of antibody titers**

Plasma titers of antibodies were determined by chemiluminescent ELISA, as previously described.16

**Clinical study**

In the observational study, a total of 183 patients visiting the Cardiovascular Risk Area of the University Clinic of Navarra (Spain) for a routine assessment were included in the study. Exclusion criteria included apparent atherosclerotic disease, based on absence of history of coronary disease, stroke, or peripheral artery disease, and a normal electrocardiogram; impaired renal or liver function, arteritis, and connective tissue diseases, inflammatory disease or cancer. Sample size was calculated according to the results of a previous study performed in our laboratory. Sample size was estimated using the program EPIDAT vs. 3.1, to detect differences of 0.2 ng/ml in sCD40L, with a statistical power of 90%. The local committee on human research approved the study, which was performed in accordance with the Declaration of Helsinki, and all participants gave written informed consent. All participants underwent a complete medical examination, and anthropometric measurements were taken. Blood pressure was measured using a mercury sphygmomanometer in a sitting position. The average of two measurements was considered. Subjects were classified as obese and non-obese according to body-mass index (BMI) (obese ≥ 27.5 kg/m²). High waist circumference was defined as >88 cm (women) and >102 cm (men). Serum and plasma were collected in Vacutainer tubes. Fasting
serum glucose, cholesterol, triglycerides (TG), and high-density lipoprotein (HDL) cholesterol were measured by standard laboratory techniques. Plasma C-reactive protein levels (hsCRP) were measured by immunoturbidimetry in a Roche Modular analyzer. sCD40 in plasma was determined by ELISA (R&D Systems, USA). Subcutaneous fat tissue samples were obtained from ZenBio (USA).

**Statistical analysis**

Data analysis in the text section is presented as mean ± standard deviation (SD). Statistical testing employed Student’s unpaired T-test between the groups as indicated. Significances in glucose tolerance (GTT) and insulin tolerance testing (ITT) were assessed by repeated two-way ANOVA between the treatment groups. In the clinical study, the normal distribution of variables was tested with the Shapiro–Wilk test. Differences across groups were compared by ANOVA, followed by the Bonferroni post-hoc test for normal variables and the Kruskal–Wallis test for non-normal variables. Spearman correlation coefficients for continuous variables were also used to assess univariate correlations. P-values <0.05 were considered statistically significant. Statistical analysis was performed with SPSS (SPSS, USA).

**Results**

**CD40 is preferably expressed by adipocytes from obese adipose tissue**

To test whether CD40 is regulated during the metabolic syndrome, we quantified levels of soluble CD40 (sCD40) in plasma of male C57BL6 mice consuming high-fat diet (HFD, 45% kcal% fat) or low-fat diet (LFD) for 20 weeks. Interestingly, sCD40 increased with HFD (133.1 ± 19.6 vs. 234.9 ± 122.9 pg/ml, n=12 per group, p=0.025, **Fig. 1A**). Accordingly, in visceral adipose tissue (VAT) lysates we detected a 137 ± 74.5 % increase in CD40 mRNA expression in
mice fed HFD (0.002 ± 0.0015 vs. 0.005 ± 0.002 copy numbers/GAPDH, n=8 per group, p=0.007, Fig. 1B). Adipocyte lysates from obese VAT, but not from lean VAT (0.15 ± 0.05 pg/ml (LFD) vs. 0.34 ± 0.08 pg/ml (HFD), n≥3 per group, p=0.01) or from cells of the stromal vascular fraction (SVF), were the main source of CD40 protein within adipose tissue (0.05 ± 0.02 pg/ml (SVF from LFD) vs. 0.07 ± 0.01 pg/ml (SVF from HFD), n≥3 per group, Fig. 1C, 1D) as previously suggested17. Within the SVF, CD8+ T cells, pro-inflammatory M1-like macrophages, and endothelial cells expressed the highest levels of CD40 (Fig. 1E). Interestingly, CD40 on CD4+ and CD8+ T cells was decreased in VAT by 74.1 ± 5.2 % (CD4+ T cells) and 40.0 ± 10.6 % (CD8+ T cells, n=5 per group, p<0.01, Fig. 1F), and to a modest degree on circulating CD8+ T cells compared with lean animals (a reduction of 9.7 ± 5.4 %, n=8 per group, p<0.01, Fig. 1G), suggesting hampered effector function of T cell CD40 in obesity.

**CD40 deficiency promotes diet-induced obesity and its metabolic complications in mice**

To test our hypothesis that CD40 signaling promotes metabolic derangements and inflammation in vivo, wild type (WT) and CD40−/− mice consumed LFD or HFD for 20 weeks. Surprisingly, CD40−/− on HFD mice gained more weight compared with age-matched WT mice (71.2 ± 18.6 (WT) vs. 95.9 ± 14.9 % starting weight (CD40−/−) at week 20 of HFD consumption, n≥19 per group, p<0.0001, Fig. 2A). This increase in weight could be attributed to enhanced fat depositions in CD40−/− mice, as shown by MRI-based body composition analysis in living animals (15.0 ± 2.5 (WT) vs. 17.3 ± 3.6 g body fat (CD40−/−) at week 20 of HFD consumption, n≥20 per group, p=0.008, Fig. 2B), and increased relative weights of peripheral fat pads (increase in CD40−/− mice by 25.9 ± 27.7 % (epididymidal, p=0.006), 18.5 ± 19.1 % (renal, p<0.05), and 78.9 ± 45.5 % (cardiac, p<0.0001), n≥8 in all groups, Fig. 2C). In accord, plasma levels of the adipokine leptin were higher in CD40-deficient mice (2.8 ± 1.1 vs. 5.3 ± 1.7 pg/ml,
n≥9 per group, p=0.001, Fig. 2D). CD40 deficiency altered properties of basic energy metabolism, such as heat production, respiratory quotient, and food intake (Suppl. Fig. S1). Despite equal changes in basic energy metabolism, CD40-deficient animals consuming LFD gained less weight (Suppl. Fig. S2, S3), suggesting that increased weight gain was diet-dependent. CD40−/− mice on HFD showed impaired glucose tolerance (Fig. 2E, n≥6 per group, p=0.0136 in repeated two-way ANOVA) and insulin resistance (Fig. 2F n≥6 per group, p<0.0001 in repeated two-way ANOVA). Likewise, fasting glucose levels were elevated in CD40−/− mice by 21.8 ± 23.3 % (n=14 per group, p<0.0001 in repeated two-way ANOVA). CD40 deficiency altered properties of basic energy metabolism, such as heat production, respiratory quotient, and food intake (Suppl. Fig. S1).

Obesity associates with hepatic steatosis19. While circulating free-fatty acids (FFAs) did not change, triglycerides and total cholesterol (Fig. 2H) rose in plasma by 25.9 ± 12.7 % (triglycerides, p=0.002, n=8 per group) and 32.0 ± 10.3 % (cholesterol, p=0.04, n=8 per group, Fig. 1I). Liver sections of CD40−/− mice showed signs of steato-hepatitis (NASH, Fig. 2J). Notably, gene regulators of lipid metabolism, such as ChREBP and ACC1 increased in CD40−/− liver tissue by 230.3 ± 116.6 % (ACC1, p<0.0001, n=14 per group,) and 688.2 ± 286.2 % (ChREBP, p<0.0001, n=14 per group, Fig. 2K).

**CD40-deficiency potentiates immune cell invasion and inflammatory gene expression in VAT**

In obesity adipose cell size increases and inflammatory cells invade into adipose tissue8. Adipocyte cell size of CD40−/− mice was significantly higher than that of WT controls (350.1 ± 104.4 (WT) vs. 387.7 ± 108.0 pixels (CD40−/−), n≥156 per group, p<0.001, Fig. 3A). Alike, SVF
of mice lacking CD40 contained more M1-like CD11c+ macrophages (3.2 ± 1.0 (WT) vs. 4.47 ± 1.3 % (CD40−/−), n≥8 per group, p<0.01) and higher numbers of CD8+ T cells (2.7 ± 1.7 (WT) vs. 6.3 ± 3.4 % (CD40−/−), n≥7 per group, p=0.02), also reflected by a higher CD8+/CD4+ T cell ratio (Fig. 3B, 3C, Suppl. Fig. S6, n≥8 per group), suggesting enhanced T cell mediated inflammation. Conversely, T-regulatory cells increased in CD40−/− VAT up to 277.8 ± 206.5 % (p=0.01, n≥8 per group). Interestingly, CD40−/− splenic CD8+ T cells, but not CD4+ T cells, expressed higher levels of the pro-inflammatory cytokine TNFα and were preferably found as activated effector-memory T cells (Suppl. Fig. S7), suggesting a pro-inflammatory T cell phenotype in CD40−/− mice. Gene array analysis confirmed elevation of various pro-inflammatory gene sets locally in VAT of CD40−/− mice (Fig. 3D), and elevated levels of the T cell chemokine RANTES (CCL5) systemically in plasma (Suppl. Fig. S8). Of note, B cell species and IgM-antibodies20 were not changed in CD40−/− mice (Suppl. Fig. S9), establishing that CD40-deficiency induces a pro-inflammatory phenotype in T-, but not in B cells.

**CD40 on lymphocytes, but not on adipocytes, accounts for its pro-inflammatory phenotype during DIO**

A variety of cell types express CD40, including leukocytes and adipocytes14, 15. To decipher which cell type contributes to the phenotype observed in CD40-deficient animals, we isolated adipocytes from CD40−/− and WT animals and quantified adipokine gene expression.

Unexpectedly, CD40-deficient adipocytes expressed similar levels of TNFα and IL-6, while MCP-1 gene expression decreased in CD40−/− mice (Supp. Fig. S10), suggesting that adipocyte CD40 does not contribute to the excessive pro-inflammatory state observed. To test whether CD40 on B- and T-lymphocytes modulates dysmetabolism and adipose tissue inflammation, we generated chimeric mice deficient for CD40 on B cells and T cells by reconstituting lethally
irradiated WT mice with mixed bone-marrow–derived cells from CD40 and RAG1−/− mice (Fig. 4A). Those Rag1/CD40 chimeras and WT mice consumed HFD for 12 weeks. Rag1/CD40 mice gained more weight (36.2 ± 9.6 (WT) vs. 51.2 ± 11.8 % starting weight (Rag1/CD40) at week 12 of HFD consumption, n≥12 per group, p=0.002, Fig. 4B) and accumulated more epididymidal (4.4 ± 1.1 (WT) vs. 5.5 ± 0.7 % body weight (Rag1/CD40), n≥11 per group, p=0.02) and perirenal fat pads (0.9 ± 0.5 (WT) vs. 1.6 ± 0.7 % body weight (Rag1/CD40), n≥11 per group, p=0.006, Fig. 4C). In addition, Rag1/CD40 chimerism deteriorated glucose (n≥5 per group, p=0.01 in repeated two-way ANOVA analysis), but not insulin tolerance (n≥5 per group, p=0.1137 in repeated two-way ANOVA analysis, Fig. 4 D-F, Suppl. Fig. S11A), promoted signs of NASH and hyperlipidemia (Fig. 4G-I), and augmented inflammatory cell accumulation — in particular that of pro-inflammatory M1-polarized macrophages (an increase by 49.4 ± 69.0 %, n≥10 per group, p=0.04, Fig. 4J, Suppl. Fig. 12). Notably, total numbers of circulating leukocytes as reservoir for infiltrating leukocytes, were not affected by Rag1/CD40 chimerism (Suppl. Fig. S11). Together, these findings support a specific role for CD40 on lymphocytes during the metabolic syndrome.

**Activation of CD40 signaling protects from adipose tissue inflammation and insulin resistance**

To test whether CD40 might be a therapeutic target in pre-established disease, 8-week-old WT mice consumed HFD for 6 weeks followed by treatment with either the stimulating anti-CD40 antibody FGK4521 or a corresponding isotype control (rat IgG2a) for additional 6 weeks. Intriguingly, activation of CD40 signaling abolished further weight gain in the FGK45 group (55.7 ± 13.5 (IgG) vs. 42.0 ± 16.9 (FGK45) weight gain as % starting weight at week 12 of HFD consumption, n≥12 per group, p=0.046, Fig. 5A). Body fat deposition decreased after FGK
treatment, as assessed by MRI whole-body fat imaging (Fig. 4B). Moreover, FGK45 treatment decreased fasting glucose levels (148.0 ± 41.7 (IgG) vs. 106.3 ± 30.3 mg/dl (FGK45), n≥11 per group, p=0.02) and improved glucose- and insulin tolerance (n≥11 per group, p=0.025 (GTT) and p=0.003 (ITT) in repeated two-way ANOVA analysis Fig. 4C-4E) and decreased insulin levels (Suppl. Fig. S13), indicating an ameliorated metabolic phenotype. Accordingly, inflammatory cell recruitment into VAT was markedly reduced as demonstrated by a decrease of the absolute number of resident VAT-leukocytes (Suppl. Fig. S14). FGK45 treatment particularly affected numbers of resident T cells, resulting in relative increases of protective CD4+ T cells (by 165 ± 44.8 %, n=11 per group, p=0.01), and a diminished CD8/CD4 ratio in adipose tissue (5.0 ± 2.7 (IgG) vs. 1.7 ± 1.9 (FGK45), n≥11 per group, p=0.003, Fig. 5F).

Relative numbers of pro-inflammatory M1-like CD11c+ macrophages fell by 53 ± 6.9% in FGK45-treated mice (n=11 per group, p=0.04, Fig. 5F, 5G). Systemically, numbers of circulating pro-inflammatory macrophages were reduced, while levels of the Th2 cytokine IL-10 increased after FGK45 treatment (Suppl. Fig. S13). These results indicate a protective role of CD40 in adipose tissue inflammation and its metabolic complications.

**Activation of CD40 signaling dampens pro-inflammatory cytokine expression in T cells**

Activation of T cells promotes adipose tissue inflammation and insulin resistance9,10,22. To explore whether CD40 participates in T cell effector function, we stimulated splenocyte cultures with FGK45. Consistent with our findings in FGK45-treated mice, numbers of CD4+, but not of CD8+ T cells, increased after anti-CD3/28 and FGK45 stimulation (Fig. 6A), indicating that proliferation of protective T cell subsets was promoted by CD40. Interestingly, stimulation of CD40 signaling did prevent T cell activation and the conversion into the T-effector-memory phenotype (Suppl. Fig. S15 A-E). Furthermore, splenic T cells treated with FGK45 expressed
lower levels of INFγ and TNFα, but not of IL-17, after activation with anti-CD3/28 (Fig. 6B, 6C) or an unspecific stimulus with ionomycin/PMA (Suppl. Fig. S15 F-G). Accordingly, FGK45 reduced TNFα expression in CD4+ (a reduction by 47.2 ± 22.8 %, n=4 per group, p=0.013) and CD8+ T cells (a reduction by 35.5 ± 9.9 %, n=4 per group, p=0.013) in ex vivo cultures of isolated SVF T cells (Fig. 6D), while SVF B cells and CD11c+ macrophages expressed higher levels of activation markers and pro-inflammatory cytokines (an increase by 81.6 ± 28.4 %, n=5 per group, p=0.002 (MCP-1/macrophages) and 44.4 ± 44.6 %, n=5 per group, p=0.045 (CD80/B cells), Fig. 6E, 6F, Suppl. Figure S16). FGK45 also decreased the levels of the pro-inflammatory mediators IL-6 and MCP-1 in the supernatant of CD8+ SVF T cells (a reduction by 48.2 ± 36.8 %, n=6 per group, p=0.01 (IL-6) and 56.3 ± 30.8 %, n=6 per group, p<0.01 (MCP-1), Fig. 6G), but did not regulate expression of TΗ-lineage defining transcription factors, such as GATA3, T-bet, and FoxP3 (Suppl. Fig. S16). Collectively, these data indicate that CD40 activation induces an anti-inflammatory T cell phenotype.

Adoptive transfer of CD40−/− T cells confers dysmetabolism and inflammation to Rag1−/− mice

To verify that T cells mediate CD40’s protective phenotype during DIO, we selectively re-populated Rag1−/− mice lacking mature B- and T cells with purified T cells from WT or CD40−/− mice, or as control with saline but without cells (Fig. 7A). All mice consumed HFD for 8 weeks. CD4+ and CD8+ T cells successfully re-populated recipient animals and were detectable in circulation and spleen (Suppl. Fig. S17). CD40-deficiency on T cells aggravated obesity (an increase of relative weight gain by 61.4 ± 76.0 %, n≥9 per group, p=0.04 and of perirenal fat pads by 39.3 ± 33.7 %, n≥9 per group, p=0.045, Fig. 7B, 7C), dyslipidemia, and increased leptin levels in donor mice (Suppl. Fig. S17). Transfer of CD40-deficient T cells impaired glucose
(n≥9 per group, p=0.03 in repeated two-way ANOVA analysis, Fig. 7D), but not insulin tolerance (n≥9 per group, p=0.42 in repeated two-way ANOVA analysis, Fig. 7E) and potentiated inflammatory cell recruitment to VAT, in particular of F4/80+ macrophages (an increase by 51.7 ± 53.0 %, n≥9 per group, p=0.03) and of CD8+ T cells macrophages (an increase by 299.7 ± 191.3 %, n≥6 per group, p=0.003, Fig. 7F, Suppl. Fig. S18). Notably, CD40−/− T cells showed higher percentages of activated effector-memory T cells (55.4 ± 9.1 (WT) vs. 70.4 ± 8.9 % (CD40−/−), n≥8 per group, p=0.003), while naïve T cells were found in lower quantities (3.7 ± 1.4 (WT) vs. 1.9 ± 1.0 % (CD40−/−), n≥8 per group, p=0.001, Fig. 7G, 7H). These data establish that CD40−/− T cells accumulate in adipose tissue and confer dysmetabolism and inflammation.

**CD40 associates with obesity in humans**

To test for relevance of our findings in humans, we quantified CD40 mRNA transcripts in lysates of subcutaneous adipose tissue. CD40 mRNA correlated with body-mass index (BMI) of donors (n=14, r=0.602, p=0.023, Fig. 8A). To attain further insight into whether CD40 expression associates with components of the metabolic syndrome, we performed a pilot study measuring sCD40 in plasma of 183 patients with a high incidence of the metabolic syndrome (31.1 %), Suppl. Table S1). Notably, we detected higher levels of soluble CD40 in the plasma of obese individuals with a high body mass index (BMI, an increase by 19.1 ± 96.3, n≥32 per group, n=0.034, Fig. 8B) and high waist circumference (WC, an increase by 20 ± 23.1, n≥80 per group, n=0.014, Fig. 8C). Notably, the association of higher sCD40 plasma levels in obese patients with a high waist circumference (Suppl. Table S2) was independently of age, gender, hypertension, glucose levels, and dyslipidemia in multivariate analysis (beta=0.20, p=0.04, Fig. 8B, 8C). In the tested cohort, sCD40 levels above the median were also independently associated with higher
levels of the adipokine leptin (13.2 ± 15 vs. 19.3 ± 20, beta=0.02, p<0.01, Fig. 8D).

Discussion

Immune cell accumulation and expression of pro-inflammatory cytokines and chemokines characterize adipose-tissue inflammation\(^{10}\). Current concepts attribute the regulation of adipose tissue inflammation to the composition of local immune cell subsets. Several reports highlighted the potential contribution of specific antigen recognition by immune cells in adipose tissue inflammation\(^{5,9,10}\). The afferent limb of adaptive immunity requires co-stimulation in addition to antigens. Indeed, we and others previously demonstrated a requirement for the co-stimulatory molecules CD40L and 4-1BB to maintain adipose tissue inflammation in mice\(^{16,23,24}\).

Surprisingly, Guo et al. have previously suggested a protective role for the CD40 receptor in adipose tissue inflammation. In their study, CD40\(^{-/-}\) mice exhibited a hyper-inflammatory phenotype with increased immune cell accumulation, elevated cytokine levels, hepatic steatosis, insulin resistance, and glucose intolerance\(^{18}\). However, whether the observed phenotype depends on CD40 expressed in the vascular bed, as suggested in this study, or on leukocyte CD40 remains unclear. In our study, we confirmed the metabolic and hyper-inflammatory phenotype of CD40\(^{-/-}\) mice and extended previous work by using various cell type specific approaches, narrowing down CD40’s phenotype to a T cell restricted effect. In line with this, CD40-deficient T cells conferred the hyper-inflammatory phenotype to lymphocyte-free Rag1\(^{null}\) mice, proposing a co-inhibitory function of CD40 receptor on adipose tissue T cells, which was responsive to pharmacological activation.

In light of CD40’s reported strong pro-inflammatory role in inflammatory disease and immunity\(^{14,25}\), it is striking that CD40 activation inhibited disease progression in a model of diet-
induced obesity. Although inflammation contributes to insulin resistance and obesity in mice and humans, the role of some key inflammatory molecules remains controversial. Schreyer et al. showed that at least one other member of the TNF-receptor superfamily, Tnfrsf1a or Tnfrsf1b, figures importantly in protecting glucose homeostasis. Accordingly, genetic deletion of both receptors predisposed to DIO. This finding is of particular interest because genetic deletion of the ligand TNFα protects from DIO. Similarly, we showed that loss of CD40L protected from DIO, while genetic loss of its receptor aggravated metabolic disease. CD40L’s diverse functions can result from engagement of different, functionally distinct receptors. Concordant with this concept, we previously reported that CD40L promotes atherogenesis, while lack of CD40 does not affect this disease. During atherogenesis, CD40L bypasses interaction with CD40 and exerts its atherogenic actions by binding the leukocyte integrin Mac-1.

In our study, loss- and gain-of function experiments support the involvement of CD40 on T cells in adipose tissue inflammation. T cells increase in obese adipose tissue in mice and humans. There, T-helper cells predominantly exhibit markers of TH1-driven activated CD44+ CD62L- effector-memory T cells. IFNγ secreted by these cells induces the expression of pro-inflammatory mediators, such as IP-10, MCP-1, and RANTES. IFNγ deficiency attenuated adipose tissue inflammation, emphasizing a pro-inflammatory role of TH1 CD4+ cells. Additionally, CD8+ T cells directly promote monocyte differentiation and macrophage activation as observed in later stages of obesity. Our data show that stimulation of both, CD4+ and CD8+, T cells with an agonistic CD40 antibody limits the production of the pro-inflammatory cytokines IFNγ and TNFα, favoring a protective phenotype in T cells and attenuating CD8+ effector function. In the classical concept of co-stimulation, CD40 ligation is required to efficiently activate B cells. However, CD40 has also been shown to provide co-stimulation to T cells,
promoting T cell proliferation and attenuating pro-inflammatory cytokine release. In line with this concept, activation of CD40 on T cells has been shown to be part of a novel co-inhibitory suppression loop by which CD40L-expressing cells, such as B cells, inhibited over-activation of T cells by abrogating pro-inflammatory and promoting protective signaling cascades. Thus, presence of CD40L-expressing cells, such as B cells or T cells, might be required to stimulate CD40 signaling and to activate this protective signaling cascade. We propose a model in which CD40 expression on both, CD4\(^+\) and CD8\(^+\), cells is required to maintain a protective milieu in lean adipose. In obesity, CD40 expression on T cells decreases and this down-regulation might cause over-activation of T cells and fuel adipose tissue inflammation and its metabolic consequences. It is unresolved whether the decrease of CD40 on T- and B cells is a result of an obesity-driven event or of more frequent CD40-CD40L interactions locally in VAT and subsequent shedding of CD40. Furthermore, CD40 deficiency favored pro-inflammatory TEM-cells and diminished the T-central memory and naïve T cells. Whether this was primarily caused by a defective T\(_{CM}\) generation as previously shown needs to be explored in future experiments. Collectively, our data indicate that the expression of pro-inflammatory cytokines in T cells, such as IFN\(\gamma\) and TNF\(\alpha\), is CD40-dependent, while neither classical T\(_{H}\)-polarizing cytokines, such as IL-10 or IL-12, nor T\(_{H}\)-lineage defining transcription factors are modulated as a direct T cell response of CD40 stimulation. In this regard, it is of note that the role of CD40 signaling in T\(_{reg}\) physiology in general and in our model remains unresolved. Guo et al have proposed compensatory proliferation of T\(_{reg}\). It is also tempting to speculate whether enhanced recruitment of T cells into VAT proceeds by activation of the RANTES/CCR5 axis as our data suggest and as was previously confirmed in DIO.

Our experiments do not ultimately exclude the primary or indirect participation of CD40
expressed on cells other than T cells. Importantly, participation of three major CD40-expressing cell types, B cells, macrophages, and adipocytes, could be ruled out: (1) Stimulation of B cells and macrophages with FGK45 increased cell activation, pro-inflammatory cytokine and chemokine secretion. Additionally, B cell subsets and antibody production were not changed throughout our study; (2) CD40-deficient adipocytes showed impaired gene expression for MCP-1, thus not contributing to the pro-inflammatory phenotype in CD40−/− mice. Also, it may be possible that minor leukocyte subsets recently identified in VAT10, but not quantified in our study, such as neutrophils, eosinophils, mast cells, or NK-cells contribute to CD40’s phenotype.

Despite CD40’s protective effect on adipose tissue inflammation, its impact on obesity remains controversial. While we show a clear effect on diet-induced weight gain in various models, a recent study reported hyper-inflammation in fat, but normal weight of CD40−/− mice18. This is interesting, since CD40−/− mice fed a low-fat diet showed lower weight gain but similar patterns of basic energy metabolism compared with HFD. This raises the question whether specific diet components might account for differential effects in the same knock-out strain.

Indeed, saturated lipid species were more frequent in the diet in our study and might be involved in dissociating obesity, insulin resistance and inflammation as recently suggested37. Also, whether different genetic backgrounds of CD40−/− animals frequently tested, such as the C57Bl6/Ncr background in our study, and the more obesity- and insulin resistance prone C57Bl/6J mice38, which served as control in our study, are a reason for the distinct inflammatory and metabolic phenotypes needs to be clarified. It is also challenging to explore how CD40 dissects increased hepatic steatosis from normal hepatic insulin resistance, but elevated expression of the transcription factor ChREBP may be a key as previously postulated39.

Targeting CD40 has the potential of modulating various human diseases. Notably, CD40
stimulation can have protective actions in various experimental inflammatory diseases — including lupus and rheumatoid arthritis\textsuperscript{21, 40} Activating anti-CD40 antibodies are currently under investigation in cancer, for the prevention of transplant rejection, and autoimmune disease\textsuperscript{41}. To investigate the relevance of our findings to humans, we measured plasma levels of soluble CD40 (sCD40), a shed product of membrane-bound CD40. Interestingly, we observed that sCD40 expression associates with clinical obesity and plasma biomarkers of obesity, such as leptin. These findings support a role for CD40 in human obesity. This finding raises the important question whether sCD40 primarily serves as a marker for CD40 expression on activated cells, or functions independently by clearing CD40’s ligands, as previously hypothesized for TNF\textsubscript{a} and TNFRs\textsuperscript{42}. This question is intriguing, as ligation of CD40 limits its protein half-life on the cell surface and favors shedding of CD40, and thus accumulation of soluble CD40 in plasma\textsuperscript{34}. However, further investigations are required to clarify the exact source and function of sCD40 in general and in obesity in particular.

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**Conflict of Interest Disclosures:** None.
References:


**Figure Legends:**

**Figure 1.** CD40 is preferably expressed by adipocytes in obese adipose tissue. WT animals were fed a high-fat diet (HFD) to induce obesity or a standard diet (low-fat diet, LFD) for 20 weeks. Plasma levels of soluble CD40 were determined by ELISA (A), CD40 mRNA in lysates of visceral adipose tissue (VAT, B). Expression of CD40 in protein lysates of the stromal vascular fraction (SVF) or the adipocyte fraction was quantified by ELISA (C) and Western Blot (D). Mean expression of CD40 (MFI) was quantified on VAT leukocytes and relative change of CD40 expression induced by HFD was plotted as % of LFD (E, F), or circulating leukocytes (G). Data are presented as mean ± SD. Statistical significance was calculated by unpaired, two-tailed Student’s T-Test.
Figure 2. CD40 deficiency aggravates the metabolic syndrome in mice. WT and CD40−/− mice consumed a high-fat diet (HFD) for 20 weeks. Relative increase of body weight (% increase of starting body weight) and total body fat, as assessed by MRI-based body composition analysis, are shown for the indicated time points (A, B). Weight of peripheral fat depositions is displayed as % body weight (C). Leptin levels were determined by ELISA (D). Glucose tolerance testing (GTT, E) and insulin tolerance testing (ITT, F) were performed after intraperitoneal injection of glucose or insulin. Plasma glucose, cholesterol, free fatty acids (FAA) and triglycerides were determined in animals fasting overnight (G, H). Liver cholesterol and triglycerides were quantified in whole tissue lysates (H). Liver sections were stained with lipid specific oil-red-O (ORO) (I). mRNA of peroxisome proliferator-activated receptor alpha (PPARα), carbohydrate responsive element-binding protein (ChREBP), sterol regulatory element binding protein-1 (SREBP1c), free fatty acid synthase (FAS), acetyl-CoA-carboxylase 1 (ACC1), and apolipoprotein-B100 (ApoB100) were determined by real-time PCR in whole liver tissue (J). Data are depicted as mean ± SD. Statistical significance was calculated by repeated two-way ANOVA (E, F) or unpaired, two-tailed Student’s T-Test between the indicated groups. Asterisk indicates p<0.05.

Figure 3. Lack of CD40 potentiates immune cell invasion and inflammatory gene expression in visceral adipose tissue (VAT). WT and CD40−/− mice consumed a high-fat diet for 20 weeks. Mean adipocyte diameter was quantified by image processing software in VAT sections (A). VAT sections were stained for the T cell antigen CD8 (B). Infiltration of leukocytes in VAT was identified by flow cytometry and expressed as percentage of all VAT cells, or as ratio of CD8+/CD4+ cells (C). mRNA preparations of VAT from 3 animals per group were analyzed by
mRNA chip array. Results are displayed as heat map based on the row Z-score (F). Data are depicted as mean ± SD. Statistical significance was calculated by unpaired, two-tailed Student’s T-Test.

**Figure 4.** Lymphocyte-expressed CD40 accounts for its pro-inflammatory phenotype during DIO. 8-week-old WT mice were lethally irradiated and reconstituted with either bone-marrow–derived cells (BM) from WT mice or a mixture of BM-cells from CD40−/− and Rag1−/− mice (Rag1/CD40) (A). Mice were subsequently pair-fed with HFD for 12 weeks. Relative increase of body weight is shown in B. Weight of peripheral fat pads was quantified at the end of the study (C). Plasma glucose was determined in animals fasting overnight (D). Insulin (ITT, E) and glucose tolerance testing (GTT, F) were performed after intraperitoneal injection of glucose or insulin. Liver lipids were quantified by Oil-red-O staining of liver sections (G) and quantification of ORO-positive area (H). Triglycerides were determined in fasting animals (I). Leukocyte infiltration into adipose tissue is shown as percentage of all VAT cells (J). Data are depicted as mean ± SD. Statistical significance was calculated by repeated two-way ANOVA (E, F) or unpaired, two-tailed Student’s T-Test between the indicated groups. Asterisk indicates p<0.05.

**Figure 5.** Activation of CD40 receptor signaling protects from the metabolic syndrome. WT mice were fed a high-fat diet (HFD) for 6 weeks. Mice were subsequently treated with the stimulating anti-CD40 antibody FGK45 or a corresponding isotype control (rat IgG2a) for 6 weeks. Increase of weight is shown as % of starting body (A). Fat depositions were imaged by MRI (B). Intraperitoneal glucose (C) and insulin tolerance (D) testing was performed. Glucose levels (E) were measured after overnight starvation. VAT-leukocyte were quantified by flow
cytometry (F). Representative histograms of CD11c-expressing F4/80+ M1 macrophages are shown in (G). SAT, subcutaneous adipose tissue. Data are depicted as mean ± SD. Statistical significance was calculated by repeated two-way ANOVA (C, D) or unpaired, two-tailed Student’s T-Test. Asterisk indicates p<0.05.

Figure 6. CD40 signaling dampens pro-inflammatory cytokine release in T cells. Splenocytes were isolated from WT mice, stained with CFSE, and stimulated in the presence of anti-CD3/28 antibodies and FGK45 or an IgG control for 72 hours. CFSE loading as marker of proliferation (A), and intracellular cytokines, such as IFNγ (B) or TNFα (C) were quantified by flow cytometry. CD4+ and CD8+ T cells (D), CD19+ B cells (E) and CD11c+ macrophages (F) were isolated from VAT of obese WT mice by bead separation and incubated with IgG or FGK45 for 24 hours. Activation markers and intracellular cytokine expression are expressed as % of IgG (D-F). Relative change of cytokines in the supernatant of CD8+ T cells induced by FGK (% change of IgG treated sample) is shown in G. Data are depicted as mean ± SD. Statistical significance was calculated by unpaired, two-tailed Student’s T-Test.

Figure 7. Adoptive transfer of CD40−/− T cells transfers dysmetabolism and inflammation into Rag1−/− mice. Rag1−/− mice were injected i.p. with 5x10^6 with purified T cells from WT or CD40−/− mice, or without cells (none, A). After 12 weeks of HFD weight gain (B) and peripheral fat depositions were quantified (C). Glucose tolerance testing (GTT, D) and insulin tolerance testing (ITT, E) were performed after intraperitoneal injection of glucose or insulin. Inflammatory cell recruitment (F) and activation of T cells (G) into VAT was characterized in flow cytometry (F). T cell subsets were defined as CD62L− CD44+ effector-memory T cells (T_{EM}), CD62L+ CD44+
central-memory T cells (T_{CM}) and CD62L^{+} CD44^{-} naïve T cells (T_{naïve}). Data are depicted as mean ± SD. Statistical significance was calculated by repeated two-way ANOVA (D, E) or unpaired, two-tailed Student’s T-Test.

**Figure 8.** CD40 associates with obesity in humans. RNA from human subcutaneous fat tissue was extracted and tested for CD40 mRNA expression. Values were normalized for GAPDH and plotted for the body-mass index (BMI) of the tested individuals (A). In a clinical cohort, sCD40 levels were quantified in 183 patients and tested for association with BMI (B), waist circumference (C), and leptin (D). High waist circumference was defined as >88 cm (women) and >102 cm (men). Median sCD40 plasma level was defined as cut-off (D). Data are depicted as mean ± SD. Statistical significance was calculated as indicated in the Methods Section. P-values are indicated in each graph.
Figure 2
Figure 3

A) Adipocyte Diameter (Pixel)

B) anti-CD8
   WT          anti-CD8
   CD40⁻/⁻

C) VAT leukocytes (% all leukocytes)

D) WT       CD40⁻/⁻

Circulation
Figure 4

(A) Schematic representation of the experimental setup showing reconstitution of Rag1−/−/CD40−/− mice with WT or Rag1−/−/CD40−/− BM cells and the subsequent lethal irradiation.

(B) Graph showing the increase of weight (%) over time (weeks) for WT and Rag1−/−/CD40−/− mice. 

(C) Bar graph comparing fat pads (%BW) for WT and Rag1−/−/CD40−/− mice. 

(D) Graph showing glucose (mg/dl) levels for WT and Rag1−/−/CD40−/− mice. 

(E) Graph showing blood glucose (mg/dl) levels over time (min) for WT and Rag1−/−/CD40−/− mice. 

(F) Graph showing blood glucose (mg/dl) levels over time (min) for WT and Rag1−/−/CD40−/− mice with AUC (%).

(G) Images showing liver Oil-red-O staining for WT and Rag1−/−/CD40−/− mice. 

(H) Bar graph comparing ORO (AU) for WT and Rag1−/−/CD40−/− mice. 

(I) Graph showing triglycerides levels for WT and Rag1−/−/CD40−/− mice. 

(J) Bar graph comparing VAT leukocytes (% all leukocytes) for WT and Rag1−/−/CD40−/− mice for different cell types: CD11c+ F4/80+, CD4+, CD8+, and CD19+.
Figure 5

A. Increase of weight (%)

B. MRI fat/water separation

C. Blood glucose (mg/dl)

D. Blood glucose (mg/dl)

E. VAT leukocytes (% of all leukocytes)

F. Glucose (mg/dl)

- CD11c+ F4/80+
- CD4+
- CD8+
- CD8/CD4
- CD19+

- CD11c

- F4/80+

- IgG
- FGK45

Figure 5
Figure 6

A. Anti-CD3/28 proliferation

B. T-cell proliferation

C. TNF$\alpha^+$ (MFI)

D. IFN$\gamma^+$ CD4$^+$ cells (%)

E. IFN$\gamma^+$ CD8$^+$ cells (%)

F. CD19$^+$ CD80

G. CD11c$^+$ MCP-1

Cytokines (% of IgG)

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<tr>
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Figure 7

A. Adoptive transfer of T-cells

B. % weight increase

C. Perirenal FP (% BW)

D. Glucose (mg/dl)

E. Glucose (mg/dl)

F. VAT leukocytes (% all VAT cells)

G. Flow cytometry analysis

H. CD8+ T-cell activation (% CD8+)

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Figure 8

(A) CD40/GAPDH mRNA

(B) sCD40 (ng/ml)

(C) sCD40 (ng/ml)

(D) Leptin (ng/ml)

BMI < 27.5
BMI > 27.5
low WC
high WC

r = 0.602, p = 0.023

p = 0.03

p = 0.014

p = 0.006
Co-Inhibitory Suppression of T Cell Activation by CD40 Protects from Obesity and Adipose Tissue Inflammation in Mice

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**EXPANDED METHODS**

**Animal Protocols.** Male CD40<sup>−/−</sup> mice (B6.129P2-Cd40tm1Kik/J, Jackson, USA) on an C57BL/6J background (approximate) and aged-matched wild-type C57BL/6J (WT) mice received a high-fat diet (HFD) a standard, low-fat diet (LFD) ad libitum for 20 weeks. As indicated, male WT mice were injected three times per week (100 µg i.p. per single injections) with the agonistic anti-CD40 antibody FGK45 (Miltenyi, Germany) or an isotype control and were pair-fed with HFD for 6 weeks. After bone marrow transplantations or adoptive transfers of T-cells animals were pair-fed with HFD as indicated. Rag-1<sup>−/−</sup> mice (B6.129S7-Rag1tm1Mom/J) were from Jackson Laboratory, USA. All mice were maintained under standardized conditions (12-hour light, 12-hour dark cycle) and had either access to food and water ad libitum or defined food intake in a separate pair-feeding experiment. For pair-feeding studies, animals in both groups were provided with a defined amount of food adjusted to the lower food intake of both groups. All experimental protocols were approved by the animal ethics committee of the Alfred Medical Research and Education Precinct (AMREP), Melbourne, Australia and the local animal ethics committee at the University of Freiburg. All procedures were carried out in accordance with institutional guidelines.

**Bone marrow transplantation.** Recipient mice were lethally irradiated with a single dose of 700cGy (Gammacell Exactor 40). Bone marrow of donor animals was filtered through a 100-mm cell strainer (BD Biosciences), centrifuged, resuspended, and injected at a concentration of 1x10<sup>7</sup> cells into the tail vein.

**Adoptive transfer of T-cells.** Splenic T-cells from WT or CD40<sup>−/−</sup> mice were isolated by negative cell separation (Stem Cell Technologies, USA). Purity was > 95 % as assessed by flow cytometry. 5x10<sup>6</sup> cells in saline were transferred into Rag-1<sup>−/−</sup> mice by intraperitoneal injection. Donor animals subsequently consumed HFD for 8 weeks. Re-population of donor animals was confirmed by detection of T-cells in flow cytometry.

**Body composition analysis.** Body composition, including total fat, lean mass, and water mass, was assessed by MRI-based body composition analysis (EchoMRI, Echo Medical Systems, USA) on a basis of four weekly follow-up measurements, or as indicated. Alternatively, MRI scans were used to image visceral fat depots.

**Intraperitoneal glucose tolerance testing (GTT) and insulin tolerance testing (ITT).** Glucose and insulin tolerance testing were performed at the indicated time points. For glucose tolerance testing (GTT), mice were deprived of food 6 hours prior to the procedures and received intraperitoneal glucose injections (0.25-1 g/kg lean body mass, as assessed by body composition analysis). For insulin tolerance testing (ITT), mice were injected with human insulin intraperitoneally (0.1-0.5 U/kg, Actrapid Insulin, Novo Nordisk, Denmark). Blood samples were collected before and 15, 30, 45, 60, 90, and 120 minutes after the insulin or glucose injection. Plasma glucose concentration was measured with AccuChek GO (Roche Diagnostics, Switzerland). Insulin plasma levels were quantified at the indicated time points by ELISA (Mecdia, Sweden).

**Euglycemic-hyperinsulinemic clamps (EHC).** After 20 weeks of diet, mice were subjected to euglycemic-hyperinsulinemic clamps. Four days prior to the clamping, mice were anesthetized and an indwelling catheter was placed into the right internal jugular vein. Mice were allowed to recover for 4 days after surgery, and body weight was monitored daily. On the day of the clamping, mice were deprived of food for 5 hours, followed by a continuous infusion of [3-3H] glucose (5 µCi bolus and 0.05 µCi/min) to measure basal glucose turnover.
After infusion of the tracer for 2 hours, EHC was performed. A continuous infusion of insulin (4 mU/kg/min) was initiated. Blood (5 µL) was sampled from the tail vein every 10 minutes to measure glucose levels. Euglycemia was maintained using a variable infusion of 30% glucose. To determine glucose specific activity, blood samples were taken regularly during the clamping. To measure tissue glucose uptake, a bolus of 2[14C] deoxyglucose (12 µCi) was injected. Blood concentrations of [3-3H] glucose and 2[14C] deoxyglucose were determined after deproteinization using BaOH and ZnSO4. Glucose uptake into individual tissues was assessed by determining the tissue content of 2[14C] deoxyglucose-6-phosphate and the plasma 2 [14C] deoxyglucose profile.

**Metabolic caging.** Animals were allowed to acclimatize to metabolic cages (Oxymax/CLAMS, Columbus Instruments, USA) for 24 hours prior to the observation period. Food intake, ambulatory movement, heat production, O2 consumption, and CO2 production were assessed and recorded during the 24-hour observation period.

**Preparation of the stromal vascular fraction (SVF).** Murine epididymidal fat pads were minced and digested with collagenase for 30 minutes at 37°C (0.5 mg/ml, Collagenase B, Roche, Switzerland). Digested tissue was then filtered through a nylon mesh (100 µm). The stromal vascular fraction (SVF) and adipocyte fraction were obtained from the resulting pellet and supernatant, respectively.

**Preparation of splenocytes.** Cells were released from spleen by nicking the capsule and gently rotating two microscope slides. Cell suspension was filtered through a nylon mesh (100 µm) and centrifuged.

**Flow cytometry.** The stromal vascular fraction (SVF) and splenocytes were obtained as described above. Remaining red blood cells were removed by incubation with a red blood cell lysing buffer (155mM NH4Cl, 5.7mM K2HPO4, 0.1mM EDTA, pH7.3). Cells were washed in PBS, and Fc-Receptors were blocked by anti-CD16/CD32 (eBioscience, USA) for 10 minutes on ice. Cells were then labeled with the indicated antibodies before quantification with a flow cytometer (BD FACS Canto II, BD Biosciences, USA). All antibodies were obtained from eBioscience, USA. Distinct leukocyte populations were identified upon cell surface expression of the indicated antigens: T-cells (CD3+), T-helper cells (CD3+CD4+CD8-), cytotoxic T-cells (CD3+CD4-CD8+), T-regulatory cells (CD4+CD25+FoxP3+), B-cells (CD19+), adipose tissue macrophages (F4/80+), pro-inflammatory, classically-activated M1-macrophages (F4/80+CD11c+), M2-macrophages (F4/80+CD11c-), dendritic cells (CD11c+), monocytes (CD11b+CD115+), inflammatory monocytes (CD11b+CD115+Gr-1+), non-inflammatory monocytes (CD11b+CD115+Gr-1+), endothelial cells (VCAM-1+).

**Isolation of total RNA and quantitative real-time PCR.** Organs were stored in RNA-stabilizing reagent (RNAlater, Qiagen, USA) after final necropsy. RNA was extracted from epididymidal adipose tissue using TRIzol Reagent (Invitrogen, USA) and glycogen as a co-precipitator (Roche, Switzerland). Homogenization was performed using a rotor-stator dispersator (IKA, Germany). 1 µg of total RNA was transcribed into cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Switzerland). Subsequent quantitative real-time PCR was performed with a LightCycler 480 System with the LightCycler 480 SYBR Green I Master (Roche, Switzerland) detection format. Murine GAPDH served as control. Primer sequences are available upon request.

**Quantification of CD40 expression.** Visceral adipose tissue was digested with collagenase as described above. The stromal vascular fraction (SVF) and the floating adipocyte fraction were separated and total protein was isolated. Equal concentrations of protein were either loaded on Western blot and stained with a specific anti-CD40 antibody and visualized or CD40 concentrations were quantified by ELISA (R&D Systems, USA).
**Microarray analysis of mRNA.** RNA samples of wild-type and CD40-deficient mice were screened using a MicroRNA V2 Expression Profiling Kit and a MouseWG-6 v2 Expression BeadChip (Illumina, Inc., USA). Samples were then analyzed on iSCAN array scanner (Illumina Inc., USA).

**Histology and immunohistochemistry (IHC).** Adipose tissue was fixed for 24 hours with 10% paraformaldehyde solution (Sigma-Aldrich, USA) at 4°C, dehydrated, embedded in paraffin, and cut into 6-µm sections. Sections were mounted on glass slides, deplanted of paraffin, and rehydrated. Sections were then placed in pre-heated Target Retrieval Solution (TRS, Dako, USA) and rinsed with PBS. For IHC staining, sections were blocked with 4% rabbit serum, incubated for 1.5 hours at RT with anti-F4/80, CD40, and CD8 (Abcam, USA), and detected by a secondary antibody, followed by detection with AEC+ substrate (DAKO, USA). Livers were embedded in Tissue-Tek compound (Sakura Finetek, Japan). For Oil-Red-O staining, liver sections were fixed in 10% neutral buffered formalin (Harleco, USA) for 10 minutes, rinsed with water, submerged in 100% Polypropylene glycol (Fischer Chemicals, Switzerland) for 2 minutes, incubated in Oil-Red-O staining solution (Sigma-Aldrich, USA) for 25 minutes at 60°C, and then washed in Millipore water and 0.05% Ammonia water.

**Analysis of murine plasma samples.** Plasma levels of insulin, leptin, adiponectin, and FFAs were measured by ELISA, according to the manufacturers’ protocols (Mercodia, Sweden; R&D Systems, USA; Cusabio, USA; Uscn Life Science Inc, China). Triglyceride, LDL, HDL, and total cholesterol levels of murine plasma samples were assessed by the COBAS Integra 400 plus system (Roche Diagnostics, Switzerland). Plasma cytokines were determined by cytometric bead array (CBA, BD Biosciences, USA), according to the manufacturer’s protocol.

**Liver homogenisates.** Liver samples were homogenized in 200µl PBS using a dispergator (IKA, Germany) and normalized to a protein concentration of 2.5 mg/ml. Lipids were extracted as previously described (Wolf et al., 2012). Samples were analyzed by liquid chromatography-mass spectrometry on a 4000QTRAP™ LC/MS/MS system (Applied Biosystems, USA).

**Measurement of antibody titers:** Plasma titers of antibodies were determined by chemiluminencescent ELISA, as previously described (Wolf et al., 2012).

**Quantification of intracellular cytokines.** Intracellular proteins were quantified by flow cytometry in splenocytes or cells of the stromal-vascular fraction. Cells were re-stimulated by phorbol-myristate-acetate (PMA, 81 nM), ionomycin (1.34 µM), brefeldin A (10 µM), or monensin (2 µM) in RPMI for 15 hours, or by cultivation on mouse anti-CD3 coated cell culture plates for the indicated time. Alternatively, specific leukocyte subsets were purified by magnetic bead positive isolation.

**Clinical study population.** In the observational study, a total of 183 patients visiting the Cardiovascular Risk Area of the University Clinic of Navarra (Spain) for a routine assessment were included in the study. Exclusion criteria included apparent atherosclerotic disease, based on absence of history of coronary disease, stroke, or peripheral artery disease, and a normal electrocardiogram; impaired renal or liver function, arteritis, and connective tissue diseases, inflammatory disease or cancer. Sample size was calculated according to the results of a previous study performed in our laboratory. Sample size was estimated using the programme EPIDAT vs. 3.1, to detect differences of 0.2 ng/ml in sCD40L, with a statistical power of 90%. The local committee on human research approved the study, which was performed in accordance with the Declaration of Helsinki, and all participants gave written informed consent. All participants underwent a complete medical examination, and anthropometric measurements were taken. Blood pressure was measured using a mercury sphygmomanometer in a sitting position. The average of two measurements was considered. Subjects were classified as obese and non-obese according to body-mass index (BMI)
(obese ≥ 27.5 kg/m²). High waist circumference was defined as >88 cm (women) and >102 cm (men). Serum and plasma were collected in Vacutainer tubes. Fasting serum glucose, cholesterol, triglycerides (TG), and high-density lipoprotein (HDL) cholesterol were measured by standard laboratory techniques. Plasma C-reactive protein levels (hsCRP) were measured by immunoturbidimetry in a Roche Modular analyzer. sCD40 in plasma was determined by ELISA (R&D Systems, USA). Subcutaneous fat tissue samples were obtained from ZenBio (USA).

Statistical analysis. Data analysis in the text section is presented as mean ± standard deviation (SD). Statistical testing employed Student’s unpaired T-test between the groups as indicated. Significances in glucose tolerance (GTT) and insulin tolerance testing (ITT) were assessed by repeated two-way ANOVA between the treatment groups. In the clinical study, the normal distribution of variables was tested with the Shapiro–Wilk test. Differences across groups were compared by ANOVA, followed by the Bonferroni post-hoc test for normal variables and the Kruskal–Wallis test for non-normal variables. Spearman correlation coefficients for continuous variables were also used to assess univariate correlations. P-values <0.05 were considered statistically significant. Statistical analysis was performed with SPSS (SPSS, USA).
**Supplemental Figures**

**Figure S1:** CD40 deficiency alters the basic energy metabolism under diet-induced obesity (DIO). C57BL6 mice competent (WT) or deficient for CD40 (CD40−/−) were fed a high-fat diet (HFD) for 20 weeks. Ambulatory movement (A), heat production (expressed as kCal per hour, B), respiratory exchange ratio (RER, C) and food intake (D) were determined during a 24-hour observation period in metabolic cages after 20 weeks of diet. CaH and fat indicate carbohydrate and fat metabolism (C). Plasma levels of neuro-endocrine regulators of appetite, such as Neuropeptide Y, Agouti-related peptide (AgRP), and Ghrelin were quantified by ELISA in plasma samples of fasting animals after 20 weeks of diet (E). Data are presented as mean ± SD of at least 8 animals per group. Statistical significance was calculated by unpaired, two-tailed Student's T-Test between both groups. P-values are indicated in each graph.
**Figure S2: CD40 deficiency alters the basic energy metabolism under a low fat diet (LFD).** Wildtype (WT) and CD40-deficient (CD40⁻/⁻) consumed a standard chow diet (LFD) for 20 weeks. Ambulatory movement (A), heat production (expressed as kCal per day, B), food intake (D), and respiratory exchange ratio (RER, C) and were determined during a 24-hour observation period after 20 weeks of diet. CaH and fat indicate carbohydrate and fat metabolism (C). Data are presented as mean ± SD of at least 9 animals per group. Statistical significance was calculated by unpaired, two-tailed Student’s T-Test between both groups. P-values are indicated in each graph.
Figure S3: CD40-deficiency improves diet-induced weight gain under a standard, low-fat diet (LFD). Wildtype (WT) and CD40-deficient (CD40-/-) consumed a standard chow diet (LFD) for 20 weeks. Diet-induced weight gain was expressed as relative weight gain from starting weight (A). Total body fat mass was assessed by EchoMRI body composition analysis in living animals at the indicated time points (B). Data are presented as mean ± SD of at least 20 animals per group. Statistical significance was calculated by unpaired, two-tailed Student's T-Test at each time point between both groups; a p-value < 0.05 was considered significant (indicated by asterisk).
Figure S4: CD40 deficiency decreases glucose-stimulated insulin secretion (GSIS). C57BL6 mice competent (WT) or deficient for CD40 (CD40$^{-/-}$) were fed a high-fat diet (HFD) or standard chow diet (LFD) for 12 weeks. Animals were non-fasting (A) or were fasted for 6 hours and subsequently glucose was injected intraperitoneally at a dose of 2g/kg body weight (B). Plasma samples were taken at 60 min after glucose injection and plasma insulin levels were quantified by ELISA. Data are presented as mean ± SD of at least 6 animals per group. Statistical significance was calculated by unpaired, two-tailed Student's T-Test between both groups; the p-value is indicated in the graph.
Figure S5. CD40 deficiency amplifies muscle insulin resistance in hyperinsulinemic-euglycemic clamps in vivo. C57BL6 mice competent (WT) or deficient for CD40 (CD40⁻⁻) were fed a standard diet (low-fat diet, LFD) or a high-fat diet (HFD) for 20 weeks. Hyperinsulinemic-euglycemic clamp analysis was performed by infused radioactively glucose (glucose infusion rate, GIR) to maintain euglycemic steady state under continuous insulin infusion. Rate of glucose disappearance was determined under basal (GDR) and insulin clamp conditions (Ins-GDR, A). Hepatic glucose production (HGP) and its suppression during insulin-clamp (% of basal HGP) was calculated (B). Glucose tracer uptake and mRNA expression of insulin receptor-1 (IRS-1) and glucose transporter-4 (GLUT-4) was quantified in muscle (C, D), and in different fat depots (E, F). SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue; BAT, brown adipose tissue. Data are presented as mean ± SD of at least 8 animals per group. Statistical significance was calculated by unpaired, two-tailed Student’s T-Test between both groups. P-values are indicated in each graph.
Figure S6: Infiltration with pro-inflammatory leukocytes is elevated in visceral adipose tissue (VAT) of CD40−/− mice. C57BL6 mice competent (WT) or deficient for CD40 (CD40−/−) were fed a high-fat diet (HFD) for 20 weeks. Leukocytes resident in visceral adipose tissue (VAT) were characterized by flow cytometry after digestion of VAT and staining with the indicated antibodies. T-helper cells were identified as CD4+ CD8−, cytotoxic T-cells as CD4− CD8+, B-cells as CD19+ cells. Adipose tissue macrophages were defined as F4/80+ cells, pro-inflammatory, classically-activated M1-macrophages as F4/80+ cells with co-expression of the dendritic cell marker CD11c. In the indicated, representative FACS plots T- and B-cells were pre-gated on lymphocytes, macrophage-gates were excluded from lymphocytes in FSC/SSC-scatter plot.
Figure S7: CD40-deficient T-cells show elevated pro-inflammatory cytokine secretion after T-cell specific activation. Splenic CD8+ T-cells from WT and CD40-/- mice were isolated by bead separation and cultivated for 24 hours in the presence of anti-CD3 and anti-CD28 antibodies to allow T-cell specific activation. Intracellular production of TNFα was quantified by flow cytometry (mean fluorescence intensity, MFI). Data are presented as mean of 12 animals per group (A). Splenic CD4+ and CD8+ cells were characterized by surface expression of CD62L and CD44 to quantify percentages of effector-memory T-cells (T_EM, CD44+, CD62L-), naive T-cells (T_naive, CD44-, CD62L+), and central-memory T-cells (T_CM, CD44+, CD62L+) in the indicated T-cell subsets. Data are presented as mean of 10 animals per group (B, C). Statistical significance was calculated by unpaired, two-tailed Student's T-Test at each time point between both groups; a p-value < 0.05 was considered significant as indicated in the graph.
**Supplemental Information to Wolf, Jehle, et al., CD40 and the metabolic syndrome**

**Figure S8**: CD40 deficiency alters local and systemic cytokine expression but does not impact on distribution of peripheral leukocyte subsets. After feeding of a high-fat diet (HFD) for 20 weeks, mRNA abundance of MCP-1 was quantified in RNA preparations of whole tissue lysates of visceral adipose tissue and normalized to GAPDH (A). IL-10, RANTES, and MCP-1 plasma concentrations were quantified in plasma by cytometric bead assay (CBA) in wild-type (WT) mice and CD40$^{-/-}$ mice (B). Peripheral, circulating leukocyte subsets were quantified by flow cytometry and concentration of peripheral blood counts (D). Monocytes were defined as CD11b$^+$ CD115$^+$, inflammatory monocytes by the additional expression of Gr-1 (Ly-6G/C). T-cell subsets were identified by FSC/SSC properties and the expression of CD3, CD4, and CD8 as indicated. B-cells were defined as CD19$^+$ cells in the lymphocyte gate. Data are presented as mean ± SD of at least 8 animals per group. Statistical significance was calculated by unpaired, two-tailed Student's T-Test between both groups; p-values are indicated in the graphs.
Figure S9. CD40 deficiency does not shift B-cell subpopulations towards a pro-inflammatory phenotype. C57BL6 and CD40−/− mice consumed a high-fat diet (HFD) for 20 weeks. B-regulatory cells, defined as CD1d+ CD5+ CD19+ lymphocytes in flow cytometry, were quantified in spleen cell suspension (A). B1a (CD5+ CD23− CD11b+ CD19+), B1b (CD5− CD23− CD11b+ CD19+), and B2-cells (CD5− CD23+ CD11b− CD19+) were identified in the peritoneal cavity (B, C). IgM-antibodies specific for the indicated epitopes were quantified in plasma samples. Data are presented as mean ± SD of at least 8 animals per group. Statistical significance was calculated by unpaired, two-tailed Student’s T-Test between both groups; a p-value < 0.05 was considered significant (indicated by asterisk).
**Figure S10. CD40 deficiency does not induce a pro-inflammatory phenotype in adipocytes.** C57BL6 mice competent (WT) or deficient for CD40 (CD40−/−) were fed a high-fat diet (HFD) for 20 weeks. Adipose tissue was digested and adipocytes were identified as floating cells after centrifugation of the cell suspension. Adipocyte mRNA abundance of the indicated genes was quantified by qPCR and normalized to GAPDH. Data are presented as mean ± SD of at least 8 animals per group. Statistical significance was calculated by unpaired, two-tailed Student's T-Test between both groups; a p-value < 0.05 was considered significant (indicated by asterisk).
Figure S11: Metabolic and inflammatory baseline characteristics of CD40-deficient lymphocytes (Rag1/CD40). 8-week-old WT mice were lethally irradiated and reconstituted with either bone-marrow–derived cells (BM) from WT mice or a mixture of BM-cells from CD40−/− and Rag1−/− mice (Rag1/CD40) to generate mice with a specific deletion of CD40 on lymphocytes. Mice were subsequently pair-fed with HFD for 12 weeks. Insulin levels were quantified in plasma of fasting and non-fasting mice after 12 weeks of HFD-consumption (A). Plasma levels of cholesterol, triglycerides, free fatty acids (FFAs), leptin, and adiponectin were determined after overnight starvation by ELISA and are expressed as % of plasma levels WT mice (B). Absolute numbers of circulating leukocyte subsets (per µl blood) were quantified by flow cytometry (C). Monocytes were identified as CD11b+CD115+, inflammatory monocytes as Gr-1+CD11b+CD115+, B-cells as CD19+, T-helper cells CD4+CD8−, and cytotoxic T-cells as CD4−CD8+ cells. Data are presented as mean ± SD of at least 11 animals per group. Statistical significance was calculated by unpaired, two-tailed Student’s T-Test at each time point between both groups; a p-value < 0.05 was considered significant.
Figure S12: Deficiency of CD40 on lymphocytes (Rag1/CD40) favors accumulation of macrophages and their polarization towards the M1-phenotype. 8-week-old WT mice were lethally irradiated and reconstituted with either bone-marrow–derived cells from WT mice or a mixture of BM-cells from CD40<sup>-/-</sup> and Rag1<sup>-/-</sup> mice (Rag1/CD40) to generate mice with a specific deletion of CD40 on lymphocytes. Mice were subsequently pair-fed with HFD for 12 weeks. Leukocytes resident in visceral adipose tissue (VAT) were characterized by flow cytometry after digestion of VAT and staining with the indicated antibodies. T-helper cells were identified as CD4<sup>+</sup>CD8<sup>-</sup>, cytotoxic T-cells as CD4<sup>-</sup>CD8<sup>+</sup> (left panel), B-cells as CD19<sup>+</sup> cells (middle panel). Adipose tissue macrophages were defined as F4/80<sup>+</sup> cells, pro-inflammatory, classically-activated M1-macrophages as F4/80<sup>+</sup> cells with co-expression of the dendritic cell marker CD11c (right panel). In the indicated, representative FACS plots T- and B-cells were pre-gated on lymphocytes, macrophage-gates were excluded from lymphocytes in FSC/SSC-scatter plot.
Figure S13: Activation of CD40 receptor ameliorates metabolic parameters and inflammatory monocytosis during DIO. 8-week-old C57BL6 mice were fed a high-fat diet (HFD) for 6 weeks to induce the metabolic syndrome. Mice were subsequently divided in two groups and treated with the stimulating anti-CD40 antibody FGK45 or a corresponding isotype control (rat IgG2a) during an additional pair-feeding with HFD for 6 weeks. Insulin levels were quantified in plasma of fasting mice after 12-weeks of HFD consumption (A). Plasma levels of cholesterol, triglycerides, free fatty acids (FFAs), leptin, and adiponectin were determined after overnight starvation by ELISA (B). Absolute numbers of circulating leukocyte subsets (per µl blood) were quantified by flow cytometry (C), and demonstrated significant reduction of total monocytes (CD11b+ CD115+), Gr-1+ inflammatory monocytes, and total B-cells (CD19+). T-helper cells were identified as CD4+ CD8- cells. Plasma level of the Th2-cytokine IL-10 was quantified by cytometric bead assay (CBA, D). Data are presented as mean ± SD of 10 animals per group. Statistical significance was calculated by unpaired, two-tailed Student’s T-Test at each time point between both groups; a p-value < 0.05 was considered significant (indicated by asterisk or by p-value in the graph).
Figure S14: Activation of CD40 signaling decreases pro-inflammatory leukocyte accumulation in visceral adipose tissue (VAT). 8-week-old C57BL6 mice were fed a high-fat diet (HFD) for 6 weeks to induce the metabolic syndrome. Mice were subsequently divided in two groups and treated with the stimulating anti-CD40 antibody FGK45 or the corresponding isotype control (rat IgG2a) during an additional pair-feeding with HFD for 6 weeks. VAT was digested and infiltration with leukocyte subsets was quantified in flow cytometry (A). T-helper cells were identified as CD4+CD8-, cytotoxic T-cells as CD4-CD8+, B-cells as CD19+ cells. Adipose tissue macrophages were defined as F4/80+ cells, pro-inflammatory, classically-activated M1-macrophages as F4/80+ cells with co-expression of the dendritic cell marker CD11c. Total number of VAT-leukocytes (B) and M1-macrophages (C) is expressed as number of cells / g adipose tissue. Data are presented as mean of at least 11 animals per group. Statistical significance was calculated by unpaired, two-tailed Student’s T-Test at each time point between both groups; a p-values are indicated in the graph. In the indicated, representative FACS plots (A) T- and B-cells were pre-gated on lymphocytes, macrophage-gates were excluded from lymphocytes in FSC/SSC-scatter plot.
Figure S15: Activation of CD40 signaling dampens CD4+ T-cell activation of splenocytes. C57BL6 mice consuming a high-fat diet (HFD) were treated with the stimulating anti-CD40 antibody FGK45 or a corresponding isotype control (rat IgG2a) for 6 weeks. CD4+ T-cells from spleen were characterized by flow cytometry, and percentages of naïve T-cells (T naïve) and effector-memory cells (T EM) were quantified (A, B). Surface expression of KLRG-1 served as a marker for senescent T-cells (C), and KI-67 served as a marker for cell proliferation (D). Activation of T-cells was evaluated by quantification of CD11a positive cells (E) and CD4+ specific intracellular expression of the cytokines INFγ, TNFα, and IL-17 after ex vivo stimulation (stim.) with ionomycin and PMA (F, G). Data are presented as mean of at least 6 animals per group. Statistical significance was calculated by unpaired, two-tailed Student’s T-Test at each time point between both groups; a p-value < 0.05 was considered significant (indicated by asterisk).
Figure S16: Activation of CD40 signaling does not regulate T-helper cell lineage defining transcription factors. CD11c⁺ dendritic cells/macrophages (A) and CD19⁺ B-cells (B) were isolated from visceral adipose tissue of HFD-consuming mice by bead isolation and stimulated with the CD40-activating antibody FGK45 or an according isotype antibody. After 24 hours of incubation, cell supernatant was screened for cytokine levels by cytometric bead assay (CBA). Cytokine levels are presented as % of IgG-stimulated concentration in the same animal. T-cells were isolated from 8-week old, male C57BL6 mice by bead separation. A purity of >90% T-cells was achieved. T-cells were subsequently stimulated by the agonistic antibody FGK45 or an according isotype control (IgG) in vitro for 24 hours. T-cell specific activation was provided by anti-CD3 and anti-CD28 monoclonal antibodies. Expression of transcription factors GATA Binding Protein 3 (GATA3, C), T-box transcription factor TBX21 (T-bet, D), and forkhead box P3 (FoxP3, E) was quantified by intracellular FACS staining. Expression is expressed as mean fluorescence intensity (MFI) for each antibody binding. Data are presented as mean ± SD of at least 4 animals per group. Statistical significance was calculated by unpaired, two-tailed Student’s T-Test at each time point between both groups; a p-value < 0.05 was considered significant as indicated in the graphs.
Figure S17. Repopulation of Rag1−/− with CD40-deficient T-cells transfers an aggravated metabolic and inflammatory phenotype. Rag-1−/− mice lacking mature B- and T-cells were injected i.p. with 5x10^6 isolated CD3+ T-cells competent (WT) or deficient for CD40−/−. Rag-1−/− mice receiving saline alone served as control (none). After 8 weeks of pairfeeding with HFD, numbers of circulating CD3+ T-cells (A), inflammatory monocytes (B), and percentage of CD3+ T-cells among splenocytes (C) were quantified by flow cytometry. Plasma levels of soluble metabolic markers, such as total cholesterol (Chol.), Triglycerides (Tri., D), free fatty acids (FFA, E), leptin (F), and adiponectin (G) were quantified in plasma samples of fasting mice. Data are presented as mean ± SD of at least 11 animals per group. Statistical significance was calculated by unpaired, two-tailed Student’s T-Test between both groups as indicated in the graphs.
Figure S18: Leukocyte infiltration in visceral adipose tissue of Rag1null mice is enhanced by repopulation with CD40-deficient T-cells. Rag-1null mice lacking mature B- and T-cells were injected i.p. with 5x10^6 isolated CD3^+ T-cells competent (WT) or deficient for CD40^-. Rag-1null mice receiving saline alone served as control (none). After 8 weeks of pairfeeding with HFD, VAT was digested and infiltration with leukocyte subsets was quantified in flow cytometry (A). T-helper cells were identified as CD4^+ CD8^-, cytotoxic T-cells as CD4^- CD8^+, B-cells as CD19^+ cells. Adipose tissue macrophages were defined as F4/80^+ cells, pro-inflammatory, classically-activated M1-macrophages as F4/80^+ cells with co-expression of the dendritic cell marker CD11c. In the indicated, representative FACS plots T-were pre-gated on lymphocytes, macrophage-gates were excluded from lymphocytes, B-cells were gated in the whole leukocyte population in FSC/SSC-scatter plot.
### SUPPLEMENTAL TABLES

#### Table S1: Demographic and clinical characteristics of the human study population.

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<td>CRP (ng/mL)</td>
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BMI, body-mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL-c, high-density cholesterol; LDL-c, low-density cholesterol; TG, triglycerides; CRP, C-reactive protein. Data are presented as mean ± standard deviation (SD).
Table S2: Demographic and clinical characteristics of the human study population.

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BMI, body-mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL-c, high-density cholesterol; LDL-c, low-density cholesterol; TG, triglycerides; CRP, C-reactive protein. High waist circumference was defined as >88 cm (women) and >102 cm (men). Data are presented as mean ± standard deviation (SD).
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SUPPLEMENTAL REFERENCES