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Adipocytes control haematopoiesis and inflammation through CD40 signaling

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Abstract

The co-stimulatory CD40-CD40L dyad plays an important role in chronic inflammatory diseases associated with ageing. Although CD40 is mainly expressed by immune cells, CD40 is also present on adipocytes. We aimed to delineate the role of adipocyte-CD40 in the aging haematopoietic system and evaluated the effects of adipocyte CD40 deficiency on cardiometabolic diseases.

Adult adipocyte CD40-deficient mice (AdiCD40KO) mice had a decrease in bone marrow (BM) haematopoietic stem cells (Lin'Sca'cKit', LSK) and common lymphoid progenitors, which was associated with increased BM adiposity and T-cell activation, along with elevated plasma corticosterone levels, a phenotype that became more pronounced with age.

Atherosclerotic AdiCD40KOApoE/- (CD40AKO) mice also displayed changes in the LSK population, showing increased myeloid- and lymphoid multipotent progenitors, and augmented corticosterone levels. Increased T-cell activation could be observed in BM, spleen, and adipose tissue (AT), while B-cell numbers were decreased. Although atherosclerosis was reduced in CD40AKO mice, plaques contained more activated T-cells and larger necrotic cores.

Analysis of peripheral AT in a diet-induced obesity model revealed that obese AdiCD40KO mice showed increased T-cell activation in AT and lymphoid organs, but exhibited decreased weight gain and improved insulin sensitivity, along with increased fat oxidation.

In conclusion, adipocyte CD40 plays an important role in maintaining immune cell homeostasis in BM during ageing and chronic inflammatory diseases, particularly of the lymphoid populations. Although adipocyte CD40-deficiency reduces atherosclerosis burden and ameliorates diet-induced obesity, the accompanying T-cell activation may eventually aggravate cardiometabolic diseases.
Introduction

The incidence of chronic low-grade inflammatory diseases, including the metabolic syndrome and cardiovascular disease (CVD) increases significantly with age. These diseases are perpetuated by an interplay of lipids, metabolism, immune cells, and inflammation. Despite lipid lowering medications such as statins for CVD patients, residual morbidity and mortality persist, making the search for additional therapeutics eminent. A search to find more effective treatments is echoed for the metabolic syndrome, as the world population grows more obese. Thus, controlling inflammation in age-related cardiometabolic diseases, such as obesity and atherosclerosis, will help reduce secondary risks and limit disease progression.

Adipocytes play a major role in the pathogenesis of these age-related diseases. In young, healthy individuals, the adipose tissue (AT) safely stores and metabolizes lipids. During ageing and/or over-nutrition, inadequate cellular processing of nutrients results in the activation of cellular stress pathways, dysfunction and expansion of adipocytes. In that state, adipocytes secrete adipokines, including leptin, TNFα, IL6, and CCL2, which attract numerous immune cells to the AT, thereby aggravating local- and eventually, systemic inflammation.

Besides being the prime cell type in peripheral, visceral, and subcutaneous AT, adipocytes are a central component of lymphoid and haematopoietic organs, especially of thymus and bone marrow (BM). Aging and over-nutrition lead to adiposity of these haematopoietic and lymphoid organs, which affects immune cell development, activation, and inflammation.

Adipocytes play a key role in inflammation not only through their capacity to release adipokines, but have also been reported as avid antigen presenting cells, expressing major histocompatibility complex (MHC)-II and CD1d, as well as the co-stimulatory immune checkpoint protein CD40.

Adipocytes were shown to induce antigen specific T-cell proliferation, and activate CD4+ T-cells by production of pro-inflammatory cytokines, which in part was attributed co-stimulation via the CD40-CD40L immune checkpoint. Immune checkpoints play a crucial role in activating and resolving inflammation, but exert cell-type specific roles, depending on the inflammatory environment. Deletion of the co-stimulatory immune checkpoints CD40 or CD40L has shown different effects on haematopoiesis, obesity, the metabolic syndrome, and atherosclerosis.

CD40L−/− mice that were subjected to an obesogenic diet exhibited less weight gain, improved insulin resistance, and diminished AT inflammation. Furthermore, T-cell specific CD40L-deficiency decreased atherosclerosis by affecting Th1 polarization and IFNγ production, but did not affect weight gain or insulin resistance.
The CD40−/− mice showed reduced atherosclerosis, as do macrophage and dendritic cell specific CD40-deficient mice. However, full body CD40-deficient mice displayed aggravated IR and severe AT inflammation during DIO, whereas deficiency of macrophage CD40 only causes minor obesity related metabolic dysfunction.

These findings highlight the cell-type specific roles of CD40 and CD40L in cardiometabolic disease, and underline the necessity to identify the functions in, and contributions of different CD40 and CD40L expressing cell types to aging-associated chronic inflammatory diseases.

As CD40 is expressed on adipocytes, and can modulate adipocyte-mediated immune cell activation, we hypothesize that adipocyte CD40 plays a critical role in mediating chronic low-grade inflammation associated with aging, atherosclerosis, and DIO.

**Methods**

**Animals**

Adipocyte CD40-deficient mice were generated by crossbreeding CD40fl/fl mice with AdipoQCre mice (Jackson laboratories, strain B6.FVB-Tg-1Evdr/J). For atherosclerosis development, AdipoQCreCD40fl/fl and CD40fl/fl littermates were crossbred with CD40fl/fl ApoE−/− mice, resulting in AdipoQCreCD40fl/fl-ApoE−/− and CD40fl/fl-ApoE−/− littermates.

**Study design**

Male adipoQCreCD40fl/fl (AdiCD40KO) mice and CD40fl/fl (WT) littermates were fed a standard chow diet ad libitum for 22 (n=8 vs n=8) or 52 (n=5 vs n=5) weeks, for analysis of haematopoiesis and immune cell composition.

For atherosclerosis development, female AdipoQCreCD40fl/fl -ApoE−/− (CD40KO, n=20) mice and CD40fl/fl-ApoE−/− (ApoE−/−, n=19) littermates were fed a high cholesterol diet (HCD; alto-10185 Energy 66% carbohydrates, 18% protein, and 16% fat with 0.15% cholesterol) for 11 weeks starting at 7 weeks of age. Mice had ad libitum access to food and water.

For diet-induced obesity (DIO), male AdiCD40KO mice (n=8) and WT littermates (n=8) were fed a high fat diet (HFD; SNIFF-D12492, Energy 22% carbohydrates, 24% protein, and 54% fat) for 15 weeks starting at 7 weeks of age. Concomitantly, male AdiCD40KO (n=7) and WT mice (n=7) were fed a standard fat diet (SFD; SNIFF-D12450B, Energy 65% carbohydrates, 26% protein, and 9% fat) for 15 weeks starting at 7 weeks of age. Mice had ad libitum access to food and water, body weight was monitored weekly.

For indirect calorimetric analysis (Promethion line, Sable Systems, Las Vegas, USA), male AdiCD40KO (n=8) and WT mice (n=8) were fed a SFD starting from 7 weeks of age, and had ad libitum access to food and water. At week 6 of diet, mice were single housed in
metabolic home-cages for 5 days. Food and water intake, respiration (O₂/CO₂), and locomotion were recorded in 5 min bins. After 8-weeks of SFD feeding, mice were fasted for 4 hours and intravenously injected with a solution containing [¹⁴C]deoxyglucose and lipoprotein-like particles labeled with glycerol tri[³H]oleate, as described previously²⁰, and blood was drawn from the tail vein at indicated times. Plasma clearance of the radiolabels was calculated from the estimated total plasma volume (0.04706*body weight) and expressed as the percentage of total injected dose. All experimental procedures were approved by the Ethical Committee for Animal Welfare of Amsterdam University Medical Center, location AMC (AVD1180020171666) and Leiden University Medical Center (AVD1160020173305). Additional methods are provided as supplementary data.
Results
To investigate the effects of adipocyte CD40 on the immune system and inflammation-driven age-related cardiometabolic diseases, we generated mice with an adipocyte-specific deletion of CD40 (adipoqCre-CD40^{f/f}; AdiCD40^{KO}). Wild-type littermates (CD40^{f/f}; WT) served as controls. Adipocyte specific CD40-deficiency was confirmed in epididymal adipose tissue (EpAT) of AdiCD40^{KO} mice with a ~70% reduction in CD40 expression compared to WT (Supplemental Figure 1A). No effects on body weight were observed between the 2 genotypes (Supp. Fig. 1B).

Adipocyte CD40-deficiency decreases haematopoiesis and increases T-cell activation
Adipocytes are abundantly present in haematopoietic and lymphoid organs where they closely interact with e.g. immune cells. We here investigated the effect of adipocyte CD40-deficiency on immune cell progenitors and immune cells in 22-week-old adult mice. AdiCD40^{KO} showed a decrease in Lin^−Sca^1^−^cKit^+^ (LSK) haematopoietic stem cells (HSC) and the common lymphoid progenitor (CLP) cells in BM (Figure 1A/B, Supp. Fig. 2A, Gating strategy Supp. Fig. BM). The reduction in CLPs resulted in a significant decrease in total BM B-cells (Figure 1C, Supp. Fig. 2B). The total amount of BM T-cells did not differ between genotypes, but an increase in BM effector memory T-cells (CD62L^−^CD44^+^) was observed, indicating an increased T-cell activation status in the BM of adipocyte CD40-deficient mice (Figure 1D, Supp. Fig. 2C).

Histological analysis of the BM showed minor degenerative changes in absence of adipocyte CD40, e.g., a trend for increased BM adiposity and a decrease in megakaryocytes (Supp. Fig. 2D). As T-cell development takes place in the thymus, we analyzed the effects of adipocyte CD40-deficiency on the different stages of T-cell development. We observed a slight reduction in total thymocytes in AdiCD40^{KO} mice compared to WT littermates (p=0.23, Supp. Fig. 2E, Gating strategy Supp. Fig. Thymus). Furthermore, the AdiCD40^{KO} mice showed a reduction in thymic T-cell development, with development stagnating at the double negative (DN) DN2L stage, while further DN stages till the double positive (DP) stage were decreased (Figure 1E, Supp. Fig. 2F). Although the amount DP T-cells was decreased, the output of single positive CD4^+^ and CD8^+^ T-cells was similar between genotypes (Figure 1F, Supp. Fig. 2G), indicating that negative selection on DP T-cells is decreased in AdiCD40^{KO} mice. Closely related to thymocyte selection are glucocorticoids, where glucocorticoids oppose thymocyte negative selection. Adipocyte CD40-deficiency did not result in structural changes to the adrenals (Supp. Fig. 2H), but AdiCD40^{KO} mice did display increased plasma corticosterone levels (Supp. Fig. 2H), which may be responsible for the observed decrease in thymocyte negative selection.
AdiCD40KO mice have altered immune cell composition in lymphoid organs

We furthermore investigated immune cell composition in lymphoid organs of the AdiCD40KO mice. We observed a decrease in B-cells in spleen and lymph nodes, whereas the number of CD3+, CD4+, CD8+, and regulatory T-cells was not affected (Supp. Fig. 3A.). However, just as in the BM, T-cells showed an increase in memory T-cells, whereas the naive T-cell population had decreased (Supp. Fig. 3A/B). This increase in T-cell activation could be confirmed in vitro, as 72h CD3-CD28 stimulated CD4+ and CD8+ AdiCD40KO T-cells showed increased IFNγ and IL2 production (Supp. Fig. 3C/D). We did not observe changes in the myeloid cells in spleens of adipocyte CD40-deficient mice (Supp. Fig. 3E), and the BM myeloid progenitors also didn’t show major differences (Supp. Fig. 3F). These findings indicate that adipocyte CD40-deficiency has a direct impact on haematopoiesis and lymphopoiesis, as well as T-cell activation in adult mice.

Aged Adipocyte CD40-deficient mice have less lymphoid progenitors

Ageing is associated with similar features of BM degeneration as observed in the adult AdiCD40KO mice. We therefore hypothesized that the phenotype described above would be more prominent in aged AdiCD40KO mice. Indeed, in 52-week-old AdiCD40KO mice, we observed a significant increase in BM adiposity (Figure 2A), as well as an absolute and relative decrease in LSK cells, including long-term (LT) and short-term (ST) stem cells, multipotent progenitors 1 (MPP1), MPP2, and MPP4 in AdiCD40KO mice (Figure 2B). The most pronounced decrease was observed in the MPP4 population, associated with B- and T-cell development. Ergo, aged AdiCD40KO mice had a decrease in the CLP and late CLP (Lin-CD127+CD27loCD25lo) populations (Figure 2C, Supp. Fig. 4A). Myeloid progenitors, derived from MMP3, did not show significant changes (Supp. Fig. 4B).

Adipocyte CD40-deficiency results in a compensatory increase in BM effector memory T-cells

As most progenitor subclasses were reduced in absence of adipocyte CD40, the number of BM CD45+ cells showed a slight decrease (Supp. Fig. 4C). Remarkably, the total number of BM T-cells was not affected in aged AdiCD40KO mice. Adipocyte CD40-deficiency caused a strong increase in CD4+ and CD8+ effector memory T-cells (EM T-cells, Figure 2C, Supp. Fig. 4D). Elevated corticosterone levels have been reported to mediate recruitment of memory T-cells to the BM through induction of chemokine receptor CXCR4 on T-cells, thereby promoting homing of these cells.22 AdiCD40KO mice showed increased plasma corticosterone levels compared to WT mice (Figure 2D), and increased CXCR4 expression on T-cells (Supp. Fig. 4E). This indicates that although T-cell development in BM is compromised when adipocyte CD40
is absent, EM T-cells will be retrieved to the BM, thereby warranting normal T-cell counts.

**Aged AdiCD40KO mice have a decrease in B-cells in lymphoid organs and BM**

In line with observations in adult AdiCD40KO mice, we observed an increase of EM T-cells in spleens of 52-week-old AdiCD40KO mice (Supp. Fig. 4F). An increase in activated T-cells is often accompanied by an increase in B-cell numbers, as T-cell-driven activation of B-cells is dependent on the CD40-CD40L axis. However, we observed a decrease in total B-cells, mostly explained by a decrease in immature B-cells (transitional- and follicular B-cells) in spleens of AdiCD40KO mice (Supp. Fig. 4G). Furthermore, in the BM, ProB and PreB-cells were decreased (Figure 2E, Supp. Fig. 4H), along with a significant decrease of IgG levels in BM interstitial fluid (Supp. Fig. 4I). This indicates either a decrease in haematopoietic output of, or a differentiation-defect in, B-cells from aged adipocyte CD40-deficient mice.

**Adipocyte CD40 in cardiometabolic disease: atherosclerosis**

To further investigate the role of adipocyte CD40 deficiency on haematopoiesis and lymphopoiesis in a chronic inflammatory disease, we backcrossed AdiCD40KO mice with ApoE−/− mice to obtain adipogCre-CD40fl/fl-ApoE−/−(CD40AKO) and CD40fl/fl-ApoE−/−(ApoE−/−) littermates to induce atherosclerosis. We found no major differences in body weight, plasma lipid levels including total cholesterol and VLDL, LDL, and HDL levels, or in total triglycerides levels between CD40 AKO and ApoE−/− mice fed a 0.15% HCD for 11 weeks (Supp. Fig. 5).

**Adipocyte CD40-deficiency enhances hypercholesterolemia-associated myelopoiesis and lymphopoiesis**

Under hypercholesterolemic conditions, CD40AKO mice exhibited an increase in both MPP3 (myeloid progenitors) and MPP4 (lymphoid progenitors) populations (Figure 3A), opposing findings from our normcholesterolemic AdiCD40KO mice. Monocytosis was substantiated by an increase in downstream monocyte progenitor subsets such as the common monocyte precursor (cMoP) and associated progenitors (Figure 3B, Supp. Fig. 6A). Previous studies have shown that hypercholesterolemia and/or ApoE deletion in humans and mice induce(s) BM monocytosis. Our finding suggests that adipocyte CD40 further contributes to hypercholesterolemia-induced aggravation of myelopoiesis. Downstream of the MPP4 population, early- to late CLP were also increased (Figure 3C, Supp. Fig. 6B). Furthermore, CD40AKO mice also showed T-cell activation in the BM as well as systemically (Supp. Fig. 6C-F), similar to AdiCD40KO mice (Figure 1D). These data could be confirmed in vitro after a 72h stimulation (Supp. Fig. 6G), and plasma levels of IFNγ and TNFα, related to T-cell activation, were also increased (Supp. Fig. 6H).
The thymus of CD40\textsuperscript{AKO} mice was seeded with an increased number of pre-thymocytes (cKit\textsuperscript{+}) derived from BM CLP (Supp. Fig. 6I), while thymic development and selection were altered, leading to a similar SP T-cell output as ApoE\textsuperscript{−/−} littermates (Figure 3D). This was associated with an increase in plasma corticosterone levels in CD40\textsuperscript{AKO} mice (Figure 3E). These findings underline the direct effect of adipocyte CD40 on T-cell development and activation even under hypercholesterolemic circumstances.

**Systemic immune cell composition is affected by adipocyte CD40 in atherosclerotic mice**

As we showed that adipocyte CD40-deficiency affects monocytosis and lymphopoiesis, we investigated the impact on the peripheral immune cell composition during hypercholesterolemia. Blood and spleen showed a similar number of CD45\textsuperscript{+} leukocytes in CD40\textsuperscript{AKO} and ApoE\textsuperscript{−/−} littermates (Supp. Fig. 7A), but CD40\textsuperscript{AKO} mice displayed an increase in monocytes (Figure 4A, Supp. Fig. 7B). In vitro we confirmed that the circulating monocytes were also more activated and had a greater capacity to transmigrate toward the CCL2 chemoattractant in a trans-well assay (Figure 4B, Supp. Fig. 7C). A significant decrease in B-cells was observed in spleen and circulation (Figure 4C, Supp. Fig. 7D). Similar to the observations in 22-week-old AdiCD40\textsuperscript{KO} mice (Supp. Fig. 4H), CD40\textsuperscript{AKO} mice had a decrease in developing B-cells in the BM (ProB and PreB-cells) (Figure 4D), causing a decrease in plasma IgG, while plasma IgM was similar between CD40\textsuperscript{AKO} and ApoE\textsuperscript{−/−} littermates (Figure 4E). These data show that adipocyte CD40-deficiency aggravates hypercholesterolemia-induced myelopoiesis and lymphopoiesis, while the opposite is shown for B-cell development.

**Adipocyte CD40-deficiency decreases lesion size but induces necrotic core formation**

Histological analysis of the aortic sinus showed mainly advanced lesions (fibrous cap atheromas) in both groups (Supp. Fig. 8A). CD40\textsuperscript{AKO} mice showed a significant reduction of atherosclerotic plaque size compared to ApoE\textsuperscript{−/−} littermates (Figure 5A). Although atherosclerotic lesions were smaller in CD40\textsuperscript{AKO} mice, necrotic core size had increased significantly (Figure 5B). Concordant with the increase in necrotic core size, plaque macrophage content had decreased (Figure 5C), along with a reduction in plaque T-cell content in CD40\textsuperscript{AKO} mice (Figure 5D). However, the number of TUNEL\textsuperscript{+} apoptotic cells, or apoptotic MAC3\textsuperscript{+} macrophages was not significantly different in absence of adipocyte CD40 (Supp. Fig. 8B), indicating efferocytosis was not affected in the plaques. Collagen and smooth muscle cell content did not change (Supp. Fig. 8C/D).

Flow cytometric analysis revealed a decrease in total CD45\textsuperscript{+} lymphocytes in the aortic root and arch of CD40\textsuperscript{AKO} mice (Supp. Fig. 8E). Immune cell composition, including percentages of innate cells, B-cells, and T-cells, did not change, indicating that the absolute numbers of all major aortic immune cell populations had decreased.
(Supp. Fig. 8F). However, the Ly6C monocyte population showed an increase in Ly6C\textsuperscript{high}/Ly6C\textsuperscript{low} ratio, demonstrating a more activated monocyte profile in CD40\textsuperscript{AKO} aortas (Supp. Fig. 8G/H). Additionally, CD3\textsuperscript{+} T-cells showed a more activated phenotype, with an increase in central memory T-cells (Figure 5E).

We also investigated AT surrounding the heart and aorta CD40\textsuperscript{AKO} and ApoE\textsuperscript{-/-} mice, as recent data indicated that cardiac/perivascular AT (CAT) is associated with cardiovascular disease severity\textsuperscript{26}. Flow cytometric analysis of adipocyte CD40-deficient CAT showed an increase in macrophage content (Supp. Fig. 8I). Furthermore, T-cell subsets showed increased activation (Figure 5F). Both these findings indicate more inflamed AT surrounding cardiac/perivascular tissue, which may have contributed to the increase in activated T-cells in lesions of CD40\textsuperscript{AKO} mice.

**Adipocyte CD40 in peripheral adipose tissue: Obesity & the metabolic syndrome**

As adipocytes not only play a role in BM and thymus, but certainly in peripheral AT, which significantly expands and changes with age, we examined the role of adipocyte CD40 in a diet-induced obesity (DIO) model by subjecting the adipocyte CD40-deficient AdiCD40\textsuperscript{KO} mice and WT littermates to a SFD or a HFD for 15 weeks. All mice significantly gained weight during the dietary period. However, adipocyte CD40-deficiency resulted in a ~15% reduction in weight gain in both SFD and HFD fed mice compared to WT littermates (Figure 6A). AdiCD40\textsuperscript{KO} mice had slight reductions in EpAT and liver weights, and adipocyte size was similar between genotypes (Supp. Fig. 9A). After 12 weeks of dietary intervention, glucose and insulin tolerance tests were performed. SFD fed AdiCD40\textsuperscript{KO} mice and WT littermates remained glucose tolerant, and no differences were detected between genotypes. In contrast, HFD fed mice became glucose intolerant, but AdiCD40\textsuperscript{KO} mice exhibited improved glucose tolerance compared to their WT counterparts (Figure 6B, Supp. Fig. 9B). No differences in in plasma glucose and insulin levels were observed (Supp. Fig. 9C/D). AdiCD40\textsuperscript{KO} mice had slightly reduced leptin and cholesterol levels, while plasma triglyceride levels were unaltered between genotypes (Supp. Fig. 9E-G). These data indicate that adipocyte CD40-deficiency diminishes manifestations of metabolic derangements in obese mice.

**Obese AdiCD40\textsuperscript{KO} mice have fewer immune cells in AT**

As inflammation is an important driver of insulin sensitivity, we investigated whether adipocyte CD40 affects immune cell activation status and composition within AT. Histological analysis revealed no major differences between the SFD groups. However, HFD fed AdiCD40\textsuperscript{KO} mice contained slightly less CD45\textsuperscript{+} leukocytes, MAC3\textsuperscript{+} macrophages and crown-like structures (Supp. Fig. 10A), as well as CD3\textsuperscript{+} T-cells in the EpAT, compared to WT mice (Figure 6C). Flow cytometric analysis
of the stromal vascular fraction corroborated the slight decrease in CD45+ cells in EpAT of HFD fed AdiCD40KO mice (Supp. Fig. 10B). Furthermore, just as in the aged AdiCD40KO mice and hypercholesterolemic CD40AKO mice, T-cell activation in EpAT of HFD fed AdiCD40KO mice had increased. Both naïve and EM T-cells had decreased, while CM T-cells were increased (Figure 6D). We observed similar changes in blood, with a decrease in naïve T-cells, and an increase in EM T-cells (Supp. Fig. 10C). In accordance with the increase in circulating EM T-cells, plasma of the AdiCD40KO mice showed increased IFNγ, IL4, and IL17 levels (Supp. Fig. 10D). Interestingly, we did not observe significant changes in the thymus of HFD AdiCD40KO mice compared to WT littermates (Supp. Fig. 10E).

In the BM, we found that a HFD had exacerbated the effect of adipocyte CD40-deficiency on HSCs, as LSK and CLP populations were decreased, while T-cell activation status increased (Figure 6 E/F, Supp. Fig. 10F/G), as has been observed in aged and atherosclerotic AdiCD40KO mice. In accordance with the findings in adult and atherosclerotic mice, obese mice have aggravated haematopoietic defects when adipocyte CD40 is deficient.

**Fat oxidation is increased in AdiCD40KO mice**

As our phenotype is propagated by adipocytes, and adipocytes play a major role in whole-body metabolism, we wanted to explore whether the adipocyte CD40-deficiency phenotype was impacting metabolism, and thereby inflammation and haematopoiesis. Therefore, 14-week-old mice that had been fed a SFD for 6 weeks, when body weights of AdiCD40KO and WT littermates were still comparable, were individually housed in fully automated metabolic cages. Voluntary locomotor activity and food intake were not affected by adipocyte CD40-deficiency (Figure 7A, Supp. Fig. 11A). Energy expenditure, as estimated from VO2 and VCO2 and normalized to fat-free mass27, was increased during both the (resting) light period and (active) dark period in AdiCD40KO mice (Supp. Fig. 11B), though not significant. More specifically, fat oxidation was found to be increased during the light period (Figure 7B/C), resulting in a decreased respiratory quotient (Supp. Fig. 11C-E).

After 8-weeks of SFD, mice were injected with [14C]deoxyglucose and lipoprotein-like particles containing glycerol tri[3H]oleate. Plasma clearance of the glycerol tri[3H]oleate tracer tended to be accelerated in AdiCD40KO mice, although this effect could not be related to enhanced [3H]oleate uptake by a certain metabolic organ (Figure 7C, Supp. Fig. 11F). Plasma clearance of [14C]deoxyglucose was comparable between genotypes, though the liver had significantly more uptake of [14C]deoxyglucose particles (Supp. Fig. 11G/H). These data indicate some changes in the metabolic profile of AT and liver in adipocyte CD40-deficient mice.

Furthermore, livers of AdiCD40KO mice showed decreased levels of steatosis, which we had previously also been observed in HFD fed
AdiCD40KO mice (Figure 7D). Transcriptome analysis of livers from SFD fed AdiCD40KO showed changes in cholesterol metabolism genes, including Srebp and Pparα, along with a decrease in the fatty acid / cholesterol (Cd36) receptor (Figure 7E). This seems to indicate decreased lipid/fatty acid overflow from the AT towards the liver. Indeed, transcriptome analysis of SFD fed AdiCD40KO AT revealed decreased expression levels of glucose (Glut4) and Cd36 receptors, along with a decrease in the LipE gene, which hydrolyzes stored triglycerides to free fatty acids (Figure 7F, Supp. Fig. 11I). These data indicate that adipocyte CD40-deficiency improves fatty acid turnover in AT and liver, increases fat oxidation, and results in decreased weight gain.

In conclusion, adipocyte CD40 plays an important regulatory role in BM and peripheral AT homeostasis. Absence of adipocyte CD40 aggravates BM degeneration, resulting in reduced levels of HSC and progenitor cells, mainly affecting the lymphoid population. This is accompanied by an increase in glucocorticoid levels, which causes enhanced compensatory recruitment of effector memory T-cells to the BM. Adipocyte CD40-deficiency also affected age-related cardiometabolic diseases. In atherosclerosis, deficiency of adipocyte CD40 resulted in a decreased plaque burden, but lesions had enlarged necrotic cores and contained more activated T-cells. During DIO, deficiency of adipocyte CD40 resulted in less weight gain, improved insulin sensitivity, and increased fat oxidation. Additionally, total lymphocytes in AT had decreased, but there was an increase in the number of activated T-cells.

**Discussion**

With age, people become more vulnerable to development of disease. This, in part, can be attributed to inflammaging. Metabolic inflammation brought on by nutrient excess and overnutrition accelerates disease progression. Inflammaging hampers haematopoiesis due to disruption of BM niches, limiting haematopoietic stem cell (HSC) survival, self-renewal, and differentiation. Adipocytes were found to play a major role in this process, as adipocytes accumulate and physically disrupt BM niches. Furthermore, adipogenesis of the thymus aggravates thymic involution, thereby reducing the immune system’s ability to grow the T-cell repertoire. These data reveal the indirect effects of adipocytes on the reduced immunity observed in the aged population. Our current study reveals that adipocytes have a direct role in haematopoiesis and immunity via the co-stimulatory protein CD40.

The CD40-CD40L co-stimulatory dyad plays a crucial role in many immunological processes such as T-cell activation and immunoglobulin production. We and others have shown that deletion of CD40 or CD40L on different-cell types differentially impact chronic inflammatory
disease progression. Genetic studies in both patients and mice have shown that CD40L-CD40 signaling might affect BM haematopoiesis and, thus, inflammatory disease progression. It was found that CD40L interaction with CD40 on HSCs induces CD40-TNF receptor associated factor (TRAF)6 signaling, which activated NFκB in HSCs. This closely links CD40-CD40L with HSC and BM niche stability, which the data on adipocyte CD40 in the current study corroborates.

Adipocytes are not only a depot for lipid storage and producers of satiety hormones, but also directly interact with immune cells. Leptin secreted by adipocytes induces IFNγ secretion by T-cells. IFNγ upregulates expression of MHC-II on adipocytes, thereby enhancing adipocyte-T-cell interaction and activation. Activation of adipocyte CD40 by CD40L increases the production of pro-inflammatory cytokines, upregulates CD40 expression, and increases adipocyte lipolysis. In obese patients, adipocyte CD40 mRNA levels are positively correlated with BMI, as well as gene expression of leptin and IL6. Deficiency of adipocyte CD40 could therefore improve metabolic function and inflammation in AT, as we indeed observed in AT of adipocyte CD40-deficient mice.

In adipocyte CD40-deficient mice, we observed an increase in fat oxidation. We propose that this results in a reduction of lipid accumulation in both AT and liver. A previous study found that dietary restriction in mice triggers a state of energy conservation, aimed at preserving immunity by retrieving T-cells to the BM, which was initiated by an increased release of glucocorticoids by the adrenals. Dietary restriction paradoxically increased adipogenesis inside the BM, while white AT deposits were decreased. Interestingly, when BM adipocytes were depleted in Adipoq-CreERT2×Rosa26-DTA mice, memory T-cells could no longer be retained in the BM. It was suggested that BM adipocytes are crucial for memory T-cell maintenance and survival through the supply of long-chain fatty acids. These data underline our findings, as we found increased glucocorticoid levels and more (CXCR4+) effector T-cells in BM and lymphoid organs, probably triggered by a decreased nutrient availability in CD40-deficient BM adipocytes.

It has been reported that BM adipocytes have distinct lipid metabolism compared to white AT, where BM adipocytes are more cholesterol-oriented, which is crucial for HSC metabolism. Furthermore, in BM adipocytes genes related to lipolysis are strongly reduced, while CD40-CD40L interaction is a strong inducer of lipolysis in white adipocytes. Therefore, the diversity in adipocyte subtypes, their functional adaptation to the different dyslipidemic environments and their environment-dependent nutrient providing capacities may explain the differences in BM, blood, and lymphoid organs that we observed between adipocyte CD40-deficient aged, atherosclerotic, and obese mice.
In hypercholesterolemic adipocyte CD40-deficient ApoE-/- mice, we found a decrease in lesion size, although necrotic core content was increased. We also found an increase in activated T-cells in these lesions. T-cells are significant drivers of the inflammatory responses that underlie atherogenesis and can promote necrotic core formation. Pro-inflammatory CD8+ T-cells have been found to promote the development of a vulnerable plaque, as antibody-mediated depletion of CD8+ T-cells in ApoE-/- mice reduced lipid and macrophage accumulation, apoptosis, and necrotic core content. Furthermore, cytokines produced by activated CD4+ T-cells can activate macrophage lipid uptake and apoptosis in the atherosclerotic lesion. Concomitantly, activated CD4+ T-cells in blood of CAD patients are directly correlated with unstable lesion phenotypes. From this, we conclude that the activated T-cells observed in the atherosclerotic plaque of adipocyte CD40-deficient mice are drivers of plaque instability.

To conclude, we have shown a central role for adipocyte CD40 in chronic inflammatory disease progression. However, our data are largely descriptive and the direct mechanism by which adipocyte CD40 influences cardiometabolic diseases needs to be further investigated. The plethora of AT interaction sites e.g., BM, thymus, and peripheral AT, as well as the diversity of functions warrants more in-depth studies. However, we can conclude that adipocyte CD40-deficiency increases fat oxidation, insulin sensitivity and thereby decreases weight gain. In addition, adipose CD40 has a key regulatory role in BM homeostasis. Its absence impacts BM cell composition and thereby haematopoiesis along with lymphopoiesis, resulting in increased T-cell activation, in models of age-related cardiometabolic disease.


**Figure Legends**

**Figure 1.** Bone marrow composition and thymic selection are altered by adipocyte CD40-deficiency. A Flow cytometric gating and analysis of 1/10 bone marrow (BM) Lin\(^{-}\)Sca\(^{c}\)Kit\(^{t}\) (LSK) cells of 22-week AdiCD40\(^{−}\) mice and WT littermates, B along with common lymphoid progenitors (CLP), Early CLP (Lin CD135\(^{−}\)CD127\(^{+}\)'), CLP, and Late CLP (Lin CD127\(^{−}\)CD27\(^{+}\)CD25\(^{−}\)). C Total amount of B-cells in BM. D Activation status of CD4\(^{+}\) T-cells, naive (CD62L\(^{+}\)CD44\(^{−}\)), effector memory (EM; CD62L\(^{−}\)CD44\(^{+}\)), and central memory (CM; CD62L\(^{−}\)CD44\(^{+}\)) in BM. E Early thymocyte ([cKit\(^{+}\)]) development of double negative (DN, CD25\(^{lo}\)hi CD44\(^{lo}\)hi) cells. F Selection of double positive thymocytes ([cKit\(^{−}\)]) into single positive CD4\(^{+}\) and CD8\(^{+}\) T-cells. Data are shown as mean±SD, n=8 AdiCD40\(^{−}\) mice and n=8 WT littermates. *P<0.05, **P<0.01, ***P<0.001.

**Figure 2.** Adipocyte CD40-deficiency has degenerative effects on the bone marrow. A Quantification of adipocytes per area in BM of 52-week n=5 AdiCD40\(^{−}\) mice and n=5 WT littermates, along with representative perilipin-1 staining of BM adipocytes (scale bar = 25 µm). B Flow cytometric analysis of 1/10 total number of long-term (LT), short-term (ST) HSC, and multipotent progenitors (MPP) in BM, C along with Early CLP, CLP, and Late CLP. D Activation status of CD4\(^{+}\) T-cells in BM. E Plasma corticosterone levels in pg/ml. F Flow cytometric analysis of B-cell maturation in BM, indicating ProB, PreB, and immature B-cells. Data are shown as mean±SD, n=5 AdiCD40\(^{−}\) mice and n=5 WT littermates. *P<0.05, **P<0.01, ***P<0.001.

**Figure 3.** Hypercholesterolemic adipocyte CD40-deficient mice have increased myelopoiesis and lymphopoiesis A Flow cytometric analyses of HSC and 1/10 total number of LT and ST HSC, along with MPP1-4 in n=15 CD40\(^{−}\) mice and n=15 ApoE\(^{−}\) littermates. B BM monocyte precursors, macrophage and dendritic cell progenitor (MDP), common monocyte precursor (cMoP), transitional pre-monocytes (tpmo), classical monocyte (CM), intermediate monocyte (IntM), and non-classical monocyte (NCM) (n=6x6). C Early CLP, CLP, and Late CLP in BM (n=15x15). D Selection of double positive thymocytes ([cKit\(^{+}\)]) into single
positive CD4+ and CD8+ T-cells (n=8x8). E Plasma corticosterone levels in pg/ml in n=20 CD40AKO mice and n=19 ApoE-/ littermates. Data are shown as mean±SD. *P<0.05, **P<0.01, ***P<0.001.

Figure 4. Hypercholesterolemic adipocyte CD40-deficient mice have an altered immune cell composition. A Flow cytometric analysis of Ly6C monocytes in spleen of n=14 CD40 AKO mice and n=13 ApoE-/- littermates. B Chemotactic trans-well assay, analyzed with flow cytometry, of blood monocytes isolated from the Top, the Bottom of the trans-well without chemotactant (NS), and from the bottom of the trans-well with 10ng/ml CCL2 (n=3x3). C Flow cytometric analysis of splenic CD3+ T-cells, CD19+ B-cells, and CD11b+ innate immune cells (n=14x13). D B-cell progenitors in BM (n=6x6). E Plasma IgM and IgG in pg/ml in n=20 CD40AKO mice and n=19 ApoE-/- littermates. Data are shown as mean±SD, *P<0.05, **P<0.01, ***P<0.001.

Figure 5. Atherosclerotic lesion composition is altered and lesion size is decreased in CD40AKO mice. A Histological analysis of total lesion size of aortic roots in mm2, along with area under the curve measurement (AUC), in n=20 CD40AKO mice and n=19 ApoE-/- littermates, with representative images (scale bar = 100 µm). B Percentage total necrotic core area in lesions, along with AUC (n=20x19). C Percentage macrophage (MAC3+) area in lesion. D Number of CD3+ T-cells per area in lesion. E Central memory (CM) CD4+ and CD8+ T-cells in aortic root and aortic arch (n=6x6). F Activation status of CD4+ and CD8+ T-cells in CAT (n=6x6). Data are shown as mean±SD. *P<0.05, **P<0.01, ***P<0.001.

Figure 6. Adipocyte CD40-deficient mice have decreased weight gain and more activated T-cells. A Weight gain (g) in n=7 standard fat diet (SFD) and n=8 high fat diet (HFD) fed AdiCD40KO and WT mice. B Glucose tolerance test glucose level (mmol/L) and clearance over time. C Histological quantification of total leukocytes (CD45+), macrophages (MAC3+), and T-cells (CD3+), and representative images of EpAT of HFD fed AdiCD40KO and WT mice (scale bar = 100 µm). D Flow cytometric analysis of activation status of CD4+ and CD8+ T-cells in EpAT. E 1/10 of LSK cells in BM of HFD fed AdiCD40KO and WT mice, F and CLP in BM. Data are shown as mean±SD, n=7 SFD and n=8 HFD AdiCD40KO and n=7 SFD and n=8 HFD WT littermates. *P<0.05, **P<0.01, ***P<0.001.

Figure 7. Fat oxidation is increased in AdiCD40KO mice. A Cumulative voluntary locomotion over time as observed in metabolic cage for n=8 SFD fed AdiCD40KO and WT mice. B Indirect calorimetry measurement determined total fat oxidation (kcal/h) rate and quantification during the light period. C Clearance of injected tri[3H]oleate over time as determined in blood (n=8x8). D Oil-Red-O staining for lipids in livers of n=8 SFD and n=8 HFD fed mice, with representative HFD images. E mRNA expression of liver and F EpAT metabolism genes. Data are shown as mean±SD, n=8 SFD AdiCD40KO and n=8 SFD WT littermates. *P<0.05, **P<0.01
Supplementary methods

Mice
Breeding and housing of all animals was done at the animal facility of the Amsterdam University Medical Center, location AMC, and the Leiden University Medical Center. All mice were backcrossed >10 times before inclusion in experiments. All the experimental procedures were approved by the Ethical Committee for Animal Welfare of Amsterdam University Medical Center, location AMC (AVD1180020171666) and Leiden University Medical Center (AVD1160020173305). After the selected dietary period mice were euthanized using a cocktail of ketamine (150 mg/kg) and xylazine (10mg/kg). Blood was obtained by cardiac puncture with an EDTA-coated syringe. Subsequently, after PBS perfusion, epididymal adipose tissue (EpAT), subcutaneous AT (ScAT), cardiac AT (CAT), liver, spleen, bone marrow (BM), thymus, and lymph nodes (LN) were removed and used for subsequent analyses. For atherosclerosis analysis, the heart and arterial tree were excised, fixed in 1% paraformaldehyde o/n, embedded in paraffin, and sectioned in 4 μm sections.

Glucose and insulin tolerance tests
In the DIO studies, one week prior to start and 12 weeks after the HFD and SFD, glucose tolerance tests (GTT) and insulin tolerance tests (ITT) were performed. For the GTT, 4h fasted mice were injected intraperitoneal with glucose (1mg/g body weight, Sigma-Aldrich). For the ITT, 4h fasted mice were injected intraperitoneal with insulin (1,1mU/g body weight, Sigma-Aldrich). Utilizing the tail vain, glucose levels were measured in whole blood using a glucometer (Bayercontour, Basel, Switzerland) at times indicated in the figures.

Analysis of atherosclerosis: morphometry and immunohistochemistry
Per mouse, 4 μm sections were cut over the length of 48 μm and stained using haematoxylin and eosin to visualize plaques in the aortic root. Two sections, 56 μm apart were stained with Picro-Sirius Red to visualize plaque collagen content. For other stainings, selected sections were rehydrated in xylene and a graded series of ethanol before pre-treatment with 0.3% H2O2 in methanol for 30 min, and sodium citrate antigen retrieval buffer (pH 6) at 100°C for 10 min (or, in the case of staining for CD3, TE antigen retrieval buffer, pH 9). Sections were then incubated with antibodies against Mac3 (1:100, clone M3/84, BD Biosciences), α-SMA (1:3000, clone 1A4, Sigma-Aldrich), ER-MP58 (1:200, Bio-Rad Laboratories B.V., Veenendaal, The Netherlands), or CD3 (1:100, clone 145-2C11, BD Biosciences). The primary antibody binding was detected by incubation with appropriate biotin-conjugated secondary antibodies (CD3/Mac3; E0468, Dako, Santa Clara, USA) and (α-SMA; 200-062-037, Jackson ImmunoResearch, Cambridgeshire, UK) followed by the ABC kit according to the manufacturer’s instructions (Vector Labs, Burlingame, USA). Immunoreactivity was visualized with 3,3’diaminobenzidine (DAB; Dako/Agilent, Santa Clara, USA). Tissue sections were counterstained with Mayer’s haematoxylin and mounted with Entellan (Merck, Darmstadt, Germany). Immunohistochemical staining omitting the primary antibodies served as negative controls. For assay of apoptotic cells, a HRP-DAB TUNEL kit (Abcam, Cambridge, UK) was used in accordance to the protocol. Images were recorded with a Leica DM6000 microscope with Las 4.1
software (Leica Microsystems, Wetzlar, Germany), and analysis was performed using Adobe Photoshop CS6, ImageJ, and Fiji.

**Flow cytometry analysis**
For flow cytometric analysis, EpAT, ScAT, CAT, spleen, BM, thymus, and LN were removed, rinsed in PBS and minced into small pieces. Adipose tissue was digested with Liberase (0.25 mg/mL, Roche, Woerden, Netherlands) for 45 min at 37°C. The digested samples were passed through a 70-µm nylon mesh (BD Biosciences, Breda, Netherlands). The SVF was obtained from the resulting pellet and resuspended in FACS buffer (0.5% BSA, 0.01% NaN3, and 2 mM EDTA in PBS). Erythrocytes in blood, spleen, and BM were removed by incubation with hypotonic lysis buffer (8.4 g of NH4Cl and 0.84 g of NaHCO3 per litre of distilled water). Cell suspensions were incubated with CD16/32 antibody (ThermoFisher) in FACS buffer to prevent non-specific binding of antibodies to the Fc receptor. Tissues were further stained with fluorophore-conjugated antibodies using CD4, CD138, Sca-1, CD27, CD38 (1:100, BD bioscience), CD16/32, Ly6G, CD8, CD3, CD19, CD135, CD48, CD45 (1:200, Biolegend), IgM (1:1600), Lin, CD150, CD127, CXCR4, CD34, CD11b, CD11c, F4/80, CD62L, CD44, FoxP3, IL-2, IFNγ, TNFα, B220, CD21, CD23, CD25, CD93 and Ly6C (1:100, eBioscience/ThermoFisher). A fixation/permeabilization kit (ThermoFisher) was used to perform intracellular staining according to manufacturer’s instructions. Flow cytometry was performed using the BD LSRFortessa (BD Biosciences) and analyzed using FlowJo v10.5.3 software (FlowJo LLC, Ashland, USA).

**T cell activation assay**
Splenocytes were isolated and CD4+ and CD8+ T cells were separated using magnetic cell separation strategies (MACS, Miltenyi Biotec, Leiden, The Netherlands) positive selection. Cells were cultured 1*10^5 in a 9-well dish and stained with a 5 µM staining solution CFSE (ThermoFisher), along with 2,5 µl CD3/CD28 Dynabeads (Invitrogen) per well. T cells were cultured in RPMI medium + 10% FCS + 1% P/S (ThermoFisher), with 20 µl/ml IL-2 and 50 µM β-mercaptoethanol for 48-72 hours. After stimulation cells were analyzed by flow cytometry using the BD LSRFortessa (BD Biosciences) and FlowJo v10.5.3 software (FlowJo LLC, USA).

**Monocyte migration assay**
For analysis of migration capacity Boyden chambers (Corning® 5 µm polycarbonate membrane, Sigma Aldrich) were used. All monocytes Ly6Clo-hi were isolated from erylysed blood using the Becton Dickinson FacsAria SORP Cell Sorter (BD Biosciences). 70K monocytes were plated on the transwells and medium with 1 µg/ml CCL2 or control (NS) was added to the bottom of the wells. After 4h the cells from the top and bottom of the well were analyzed using the BD LSRFortessa (BD Biosciences) and FlowJo v10.5.3 software (FlowJo LLC, USA).

**Plasma measurements**
Blood cholesterol and triglycerides were assessed by enzymatic colorimetric kits according to the vendor’s instructions (Roche Diagnostics, Indianapolis, USA). Mouse fasting plasma insulin levels were measured in samples from 4h fasted mice using an insulin ELISA kit according to the vendor’s instructions (Mercodia, Uppsala, Sweden). Plasma leptin and corticosterone levels (obtained at ~10
AM at sacrifice) were measured with a mouse leptin ELISA kit (ChrystalChem, Zaandam, Netherlands) and a mouse corticosterone competitive ELISA kit (ThermoFisher, Bleiswijk, Netherlands). Plasma cytokine levels were measured on a Luminex 200 system using a multiplex mouse Procartaplex panel (ThermoFisher) containing markers for IFN-γ, TNF-α, IL1β, IL2, IL4, IL5, IL6, IL10, IL12p70, IL17A, IL17F, IL23, G-CSF, IP-10, GM-CSF, KCGRO, MCP-1, MCP-3, MIP-1α, MIP-1β, and MIP-2.

Total IgM and IgG concentrations of plasma were detected using IgG and IgM mouse uncoated ELISA Kits (ThermoFisher).

Histology

Adipose tissues was collected, fixed in 4% paraformaldehyde and embedded in paraffin. Immunohistochemistry on EpAT was performed for CD45 (1:1000, BD bioscience) and MAC3 (1:100, BD bioscience), and adipocyte size was measured on EpAT H&E-stained sections. Femurs were collected, fixed in 4% paraformaldehyde, decalcified in EDTA solution for 3 weeks, and embedded in paraffin. Immunohistochemistry on femurs was performed for perilipin-1 (1:200, Abcam). Livers were embedded in OCT (Sakura, Alphen aan den Rijn, Netherlands) and frozen at -80 °C. To measure tissue lipid content, 5 μm thick cryosections of the liver and 8 μm thick cryosections of the adrenal gland were stained with Oil red O (Sigma-Aldrich, Zwijndrecht, Netherlands).

RNA isolation and gene expression analysis

Total RNA from adipose tissue, BM, and liver was extracted using TRIzol (Invitrogen, Carlsbad, USA). RNA was reverse transcribed using an iScript cDNA synthesis kit (Bio-Rad, Veenendaal, Netherlands), and quantitative PCR was performed with the SYBR Green PCR kit (Applied Biosystems, Leusden, Netherlands) on a ViiA7 real time PCR system (Applied Biosystems). Expression levels of transcripts obtained with real-time PCR were normalized to the mean expression levels of the house keeping genes Gapdh and B2m.

Adipocyte culture

Adipocytes from AdiCD40KO and WT mice were cultured by differentiating pre-adipocytes on a collagen hydrogel (Corning®, Osdorp, Netherlands). Adipose tissue isolated from mice was minced and digested with Dispase II (10mg/ml, Sigma-Aldrich) and Collagenase D (200mg/ml, Roche) for 1h at 37°C. The digested mixture was strained through a 70 µm filter and spun for isolation of pre-adipocytes. Pre-adipocytes were cultured for 5 days on rat tail collagen type 1 (100µg/ml, Corning®) pre-coated plates. Adherent pre-adipocytes were trypsinized (ThermoFisher) and diluted at 6*10^5 cells/24 well. Cells were added to the hydrogel and cultured for 2 days with cell differentiation medium (DMEM/F12 + GlutaMAX (ThermoFisher) + 10% FCS + 1% P/S, Dexamethasone (5uM, Sigma), insulin (2.5ug/mL), isobutylmethylxanthine (0.5mM, ThermoFisher), and rosiglitazone (3uM, CaymanChem, Ann Arbor, USA). Additionally, differentiated adipocytes were cultured for 7 days with DMEM/F12 + GlutaMAX + 10% FCS + 1% P/S + insulin (2.5ug/mL) before experiments or analysis.

Statistical analysis

Data are expressed as mean±SD. Differences between groups were analyzed using an unpaired t
test or Mann-Whitney U test if not conforming to Gaussian distribution as assessed by D'Agostino-Pearson omnibus normality test. A P value of <0.05 was considered significant. Outliers were identified using Grubbs' test (alpha=0.05). Statistical analysis was performed using Graphpad Prism 9.1.2.
**Supplemental Figures**

Supplemental Central Figure, haematopoietic stem cell flow cytometric markers and gating strategies for bone marrow and thymus.

**Supplemental Figure 1A** Relative mRNA CD40 expression in adipocytes derived from EpAT in AdiCD40\textsuperscript{KO} mice and WT littermates. **B** Weights of Adipocyte CD40-deficient and WT littermates at sacrifice of indicated diets. \(n=5\) 52-week old chow fed (Chow52), \(n=8\) 22-week old chow fed (Chow22), \(n=7\) standard fat diet (SFD), \(n=8\) high fat diet (HFD), \(n=8\) SFD that underwent the metabolic cage study (SFDmc).

Supp. Fig. 2A Common lymphoid progenitor (CLP) as a percentage of Lin- cells in BM of AdiCD40\textsuperscript{KO} mice and WT littermates. **B** Flow cytometric analysis of CD19\textsuperscript{+} B-cells of CD45\textsuperscript{+} cells in BM. **C** Amount of naive, effector memory, and central memory CD4 T-cells in BM. **D** Histological quantification of adipocytes and megakaryocytes per area in BM. **E** Total amount of thymocytes per gram thymus. **F** Early thymocyte (cKit\textsuperscript{+}) development of double negative (DN, CD25\textsuperscript{lo}-hi CD44\textsuperscript{lo}-hi) cells. **G** Selection of double positive thymocytes (cKit\textsuperscript{-}) into single positive CD4\textsuperscript{+} and CD8\textsuperscript{+} T-cells. **H** Plasma corticosterone levels in pg/ml. **I** Diameter of adrenal gland in \(n=5\) AdiCD40\textsuperscript{KO} mice and \(n=5\) WT littermates. **J** Percentage Oil-Red-O area in adrenal gland cross-section (\(n=5\times5\)). Data are shown as mean±SD, \(n=8\) AdiCD40\textsuperscript{KO} mice and \(n=8\) WT littermates. *\(P<0.05\), **\(P<0.01\), ***\(P<0.001\).

Supp. Fig. 3A Flow cytometric analysis of lymphoid populations in the inguinal LN. **B** Flow cytometric analysis of splenocytes isolated from WT and AdiCD40KO mice, showing naive (CD62L\textsuperscript{+}CD44\textsuperscript{-}), central memory (CD62L\textsuperscript{-}CD44\textsuperscript{+}), and effector memory (CD62L\textsuperscript{-}CD44\textsuperscript{+}) CD4\textsuperscript{+} T-cells (\(n=4\times3\)). **C** Intracellular cytokine expression of CD4\textsuperscript{+} T-cells at day 3 after stimulation with CD3/CD28 beads (\(n=4\times3\)). **D** Intracellular cytokine expression of CD8\textsuperscript{+} T-cells at day 3 after stimulation with CD3/CD28 beads (\(n=4\times3\)). **E** Flow cytometric analysis of splenocytes. **F** Flow cytometric analysis of the Lin-cKit+Sca- population presenting the myeloid lineage precursors. Data are shown as mean±SD, \(n=8\) AdiCD40\textsuperscript{KO} mice and \(n=8\) WT littermates. *\(P<0.05\), **\(P<0.01\), ***\(P<0.001\).

Supp. Fig. 4A Common lymphoid progenitor (CLP) as a percentage of Lin- cells in BM of aged 52-week old AdiCD40\textsuperscript{KO} mice and WT littermates. **B** Flow cytometric analysis of the Lin-cKit+Sca-population presenting the myeloid lineage precursors. **C** 10% of Lin\textsuperscript{-}CD45\textsuperscript{+} cells in BM. **D** Activation status of CD4\textsuperscript{+} T-cells in BM. **E** CXCR4 expression on splenic CD4\textsuperscript{+} and CD8\textsuperscript{+} T-cells. **F** Activation status of CD4\textsuperscript{+} T-cells in spleen. **G** Transitional (T1/T2) B-cells and follicular B-cells in spleen. **H** Flow cytometric analysis of B-cell maturation in BM, indicating ProB, PreB, and immature B-cells. **I** Interstitial BM IgG in pg/ml. Data are shown as mean±SD, aged \(n=5\) AdiCD40\textsuperscript{KO} mice and \(n=5\) WT littermates. *\(P<0.05\), **\(P<0.01\), ***\(P<0.001\).
**Supp. Fig. 5A** Weights (g) of CD40KO mice and ApoE⁻/⁻ littermates after 11 weeks of high cholesterol diet. **B** Plasma total cholesterol (tCHOL), VLDL, LDL, and HDL subtypes, along with **C** plasma triglycerides (TRIG), free fatty acids (free glycerol) and VLDL, LDL, HDL subtypes in CD40KO mice and ApoE⁻/⁻ littermates. Data are shown as mean±SD, n=20 CD40AKO mice and n=19 ApoE⁻/⁻ littermates. *P<0.05

**Supp. Fig. 6A** Flow cytometric analysis of BM monocyte precursors, macrophage and dendritic cell progenitor (MDP), common monocyte progenitor (cMoP), transitional pre-monocytes (tmo), classical monocyte (CM), intermediate monocyte (IntM), and non-classical monocyte (NCM) (n=6x6). **B** Early CLP, CLP, and Late CLP in BM (n=15x15). **C** Flow cytometric analysis of activation status of CD4⁺ T-cells, naïve (CD62L⁺CD44⁻), effector memory (EM; CD62L⁻CD44⁺), and central memory (CM; CD62L⁻CD44⁺) in BM. **D** Activation status of CD4⁺ T-cells in blood. **E** Activation status of CD4⁺ T-cells in spleen. **F** Activation status of CD4⁺ T-cells in EpAT. **G** Expression of activation markers on CD4⁺ T-cells at day 3 after stimulation with CD3/CD28 beads (n=4x3). **H** Plasma cytokines in CD40KO mice and ApoE⁻/⁻ littermates. **I** Flow cytometric analysis of cKit⁺ thymocytes in thymus. **J** Selection of double positive thymocytes (cKit⁻) into single positive CD4⁺ and CD8⁺ T-cells (n=8x8). Data are shown as mean±SD, n=20 CD40AKO mice and n=19 ApoE⁻/⁻ littermates. *P<0.05, **P<0.01, ***P<0.001.

**Supp. Fig. 7A** Flow cytometric analysis of CD45⁺ lymphocytes in blood and spleen of CD40KO mice and ApoE⁻/⁻ littermates. **B** Flow cytometric analysis of Ly6C monocytes in blood. **C** Expression of CCR2 receptor on all CD40AKO and ApoE⁻/⁻ monocytes in trans-well (n=3x3). **D** Flow cytometric analysis of CD3⁺ T-cells, CD19⁺ B-cells, and CD11b⁺ innate immune cells in blood. Data are shown as mean±SD, n=20 CD40AKO mice and n=19 ApoE⁻/⁻ littermates. *P<0.05, **P<0.01

**Supp. Fig. 8A** Virmani score staging of atherosclerotic lesions, ix= initial xanthoma, pit= pathologic intimal thickening, fca= fibrous cap atheroma (thin and thick), in CD40KO mice and ApoE⁻/⁻ littermates. **B** Apoptotic cells in atherosclerotic roots were detected by the TUNEL (TdT-mediated dUTP nick end labeling) and area TUNEL⁺ macrophages in the atherosclerotic roots of ApoE⁻/⁻ and CD40AKO mice. **C** Percentage collagen stained by sirius red (SR) in lesions. **D** Percentage smooth muscle cell stained by ASMA⁺ in lesions. **E** Flow cytometric analysis of CD45⁺ cells in aortic root and aortic arch (n=6x6). **F** Flow cytometric analysis of CD19⁺ B-cells, CD4⁺ and CD8⁺ T-cells, and F4/80⁺ macrophages in aortic roots and aortic arches (n=6x6). **G** Ly6C monocytes of CD11b⁺ innate cells in aortic root and aortic arch (n=6x6). **H** Histological quantification of ER-MP58 early monocyte staining in aortic root lesions. **I** Flow cytometric analysis of macrophages (F4/80⁺) and Ly6C monocytes in cardiac/perivascular adipose tissue (CAT) (n=6x6). Data are shown as mean±SD, n=20 CD40AKO mice and n=19 ApoE⁻/⁻ littermates. *P<0.05
**Supp. Fig. 9A** Weights (g) of liver and epididymal adipose tissue (EpAT) of SFD fed AdiCD40KO mice and WT littermates, along with HFD fed AdiCD40KO and WT mice. **B** Area under the curve analysis of glucose tolerance test. **C** Insulin tolerance test glucose level (mmol/L) over time for SFD and HFD AdiCD40KO mice and WT littermates. **D** Plasma insulin levels after 4h fasting in SFD and HFD fed AdiCD40KO mice and WT littermates. **E** Triglycerides (mmol/L) levels in plasma of SFD and HFD fed AdiCD40KO mice and WT littermates. **F** Cholesterol (mmol/L) levels in plasma of SFD and HFD fed AdiCD40KO mice and WT littermates. **G** Leptin (ng/mL) levels in plasma of SFD and HFD fed AdiCD40KO mice and WT littermates. Data are shown as mean±SD, n=7 SFD and n=8 HFD AdiCD40KO and n=7 SFD and n=8 HFD WT littermates. *P<0.05, **P<0.01

**Supp. Fig. 10A** Average Crown-like-structures determined through MAC3+ staining in EpAT of HFD AdiCD40KO mice and WT littermates. **B** Flow cytometric analysis of CD45+ cells in 0.5 gram EpAT of HFD fed AdiCD40KO mice and WT littermates. **C** Flow cytometric analysis of activation status of CD4+ and CD8+ T-cells in blood. **D** Plasma cytokines in HFD fed AdiCD40KO mice and WT littermates. **E** Thymocyte (cKit+) development of double negative (DN, CD25lo-hi CD44lo-hi) cells in thymus of HFD mice. **F** Flow cytometric analysis of CLP in BM of HFD fed mice. **G** Activation status of CD4+ and CD8+ T-cells in BM. Data are shown as mean±SD, n=8 HFD AdiCD40KO and n=8 HFD WT littermates. *P<0.05, **P<0.01

**Supp. Fig. 11A** Cumulative food intake (g) over time as observed in metabolic cage for n=8 SFD fed AdiCD40KO and WT mice. **B** Indirect calorimetry measurement determined energy expenditure (kcal/h) during the light and dark period. **C** Volumetric oxygen intake (VO2) along with **D** volumetric carbon dioxide (VCO2) release over time determined in metabolic cage in SFD fed AdiCD40KO and WT mice. **E** Respiration quotient as determined in AdiCD40KO and WT mice. **F** Relative glycerol tri[3H]oleate uptake in different organs determined as dose/gram tissue of SFD fed AdiCD40KO and WT mice. **G** Clearance of injected [14C]deoxyglucose over time as determined in blood. **H** Relative [14C]deoxyglucose uptake in different organs determined as dose/gram tissue of SFD fed AdiCD40KO and WT mice. **I** STRING interaction network result for metabolic genes in adipose tissue and liver. The interactome can be observed, as well as relative fold-change in expression (low fold-change is light, high fold-change is blue), along with the grouping by the STRING software of clustered genes (https://string-db.org/, figure obtained 22-09-2022). Data are shown as mean±SD, n=8 SFD AdiCD40KO and n=8 SFD WT littermates. *P<0.05
Gating strategies
Bone marrow

Thymus