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# Adipocytes control hematopoiesis 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 aging. 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 hematopoietic system and evaluated the effects of adipocyte CD40 deficiency on cardiometabolic diseases. Adult adipocyte CD40-deficient mice (AdiCD40<sup>KO</sup>) mice had a decrease in bone marrow hematopoietic stem cells (Lin<sup>-</sup>Sca<sup>+</sup>cKit<sup>+</sup>, LSK) and common lymphoid progenitors, which was associated with increased bone marrow adiposity and T-cell activation, along with elevated plasma corticosterone levels, a phenotype that became more pronounced with age. Atherosclerotic AdiCD40<sup>KO</sup>ApoE<sup>-/-</sup> (CD40<sup>AKO</sup>) 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 bone marrow, spleen, and adipose tissue, while the numbers of B cells were decreased. Although atherosclerosis was reduced in CD40<sup>AKO</sup> mice, plaques contained more activated T cells and larger necrotic cores. Analysis of peripheral adipose tissue in a diet-induced model of obesity revealed that obese AdiCD40<sup>KO</sup> mice had increased T-cell activation in adipose tissue and lymphoid organs, but 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 bone marrow during aging 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, increases significantly with age.<sup>1</sup> These diseases are perpetuated by an interplay of lipids, metabolism, immune cells, and inflammation. Despite lipid-lowering medications such as statins for patients with cardiovascular disease, residual morbidity and mortality persist, making the search for additional therapeutics important.<sup>2</sup> A search to find more effective treatments is echoed for the metabolic syndrome, as the world's population grows more obese.<sup>3</sup> Thus, controlling inflammation in age-related cardiometabolic diseases,

such as obesity and atherosclerosis, will help to reduce secondary risks and limit disease progression.<sup>2,4</sup>

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 aging 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, tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), interleukin 6 (IL6), and CC-motif chemokine ligand 2 (CCL2), which attract numerous immune cells to the AT,<sup>5</sup> thereby aggravating local and, eventually, systemic inflammation.<sup>6</sup>

Besides being the prime cell type in peripheral, visceral, and subcutaneous AT, adipocytes are a central component of lymphoid and hematopoietic organs, especially of thymus and bone marrow (BM).<sup>7</sup> Aging and over-nutrition lead to adiposity of these hematopoietic and lymphoid organs, which affects immune cell development, activation, and inflammation.<sup>7</sup>

Adipocytes play a key role in inflammation not only through their capacity to release adipokines; they have also been reported to be avid antigen-presenting cells, expressing major histocompatibility complex-II and CD1d, as well as the co-stimulatory immune checkpoint protein CD40.<sup>8,9</sup>

Adipocytes were shown to induce antigen-specific T-cell proliferation, and activate CD4<sup>+</sup> T cells by production of pro-inflammatory cytokines,<sup>8</sup> which in part was attributed to co-stimulation via the CD40-CD40L immune checkpoint.<sup>8,10</sup>

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 hematopoiesis, obesity, the metabolic syndrome, and atherosclerosis.<sup>4,11-19</sup>

CD40L<sup>-/-</sup> mice that were subjected to an obesogenic diet exhibited less weight gain, improved insulin resistance, and diminished AT inflammation.<sup>13</sup> Furthermore, T-cell-specific CD40L deficiency decreased atherosclerosis by affecting Th1 polarization and interferon- $\gamma$  (IFN $\gamma$ ) production,<sup>10</sup> but did not affect weight gain or insulin resistance in mice with diet-induced obesity.<sup>14</sup> CD40<sup>-/-</sup> mice showed reduced atherosclerosis,<sup>15</sup> as did mice with CD40 deficiency specifically of macrophages<sup>17</sup> and dendritic cells.<sup>10</sup> However, mice with full body CD40 deficiency displayed aggravated insulin resistance and severe AT inflammation during diet-induced obesity,<sup>16,19</sup> whereas deficiency of macrophage CD40 only caused minor obesity-related metabolic dysfunction.<sup>18</sup>

These findings highlight the cell-type specific roles of CD40 and CD40L in cardiometabolic disease, and underscore the need to identify the functions 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,<sup>8,9</sup> we hypothesized that adipocyte CD40 plays a critical role in mediating chronic, low-grade inflammation associated with aging, atherosclerosis, and diet-induced obesity.

## Methods

### Animals

Adipocyte CD40-deficient mice were generated by cross-breeding CD40<sup>fl/fl</sup> mice<sup>10</sup> with AdipoQCre mice (Jackson Laboratories, strain B6.FVB-Tg-1Evdr/J). For atherosclerosis development, AdipoQCreCD40<sup>fl/fl</sup> and CD40<sup>fl/fl</sup> littermates

were crossbred with CD40<sup>fl/fl</sup> ApoE<sup>-/-</sup> mice,<sup>10</sup> resulting in AdipoQCreCD40<sup>fl/fl</sup>-ApoE<sup>-/-</sup> and CD40<sup>fl/fl</sup>-ApoE<sup>-/-</sup> littermates.

### Study design

Male AdipoQCreCD40<sup>fl/fl</sup> (AdiCD40<sup>KO</sup>) mice and CD40<sup>fl/fl</sup> (wildtype, WT) littermates were fed a standard chow diet *ad libitum* for 22 weeks (8 mice in each group) or 52 weeks (5 mice in each group), for analysis of hematopoiesis and immune cell composition.

For atherosclerosis development, female AdipoQCreCD40<sup>fl/fl</sup>-ApoE<sup>-/-</sup> mice (CD40<sup>AKO</sup>, n=20) and CD40<sup>fl/fl</sup> ApoE<sup>-/-</sup> littermates (ApoE<sup>-/-</sup>, n=19) were fed a high cholesterol diet (alto-10185, energy: 66% carbohydrates, 18% protein, and 16% fat with 0.15% cholesterol) for 11 weeks starting at 7 weeks of age. The mice had *ad libitum* access to food and water.

For diet-induced obesity, male AdiCD40<sup>KO</sup> mice (n=8) and WT littermates (n=8) were fed a high-fat diet (SSNIFF-D12492, energy: 22% carbohydrates, 24% protein, and 54% fat) for 15 weeks starting at 7 weeks of age. Concomitantly, male AdiCD40<sup>KO</sup> (n=7) and WT (n=7) mice were fed a standard fat diet (SSNIFF-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, CA, USA), male AdiCD40<sup>KO</sup> (n=8) and WT (n=8) mice were fed a standard-fat diet starting from 7 weeks of age, and had *ad libitum* access to food and water. At week 6 of the diet, mice were singly-housed in metabolic home-cages for 5 days. Food and water intake, respiration (O<sub>2</sub>/CO<sub>2</sub>), and locomotion were recorded in 5 min bins. After 8 weeks of the standard-fat diet, the mice were fasted for 4 hours and intravenously injected with a solution containing [<sup>14</sup>C]deoxyglucose and lipoprotein-like particles labeled with glycerol tri[<sup>3</sup>H]oleate, as described previously,<sup>20</sup> 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 x 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 *Online 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<sup>fl/fl</sup>;

AdiCD40<sup>KO</sup>). WT littermates (CD40<sup>fl/fl</sup>) served as controls. Adipocyte-specific CD40 deficiency was confirmed in epididymal AT of AdiCD40<sup>KO</sup> mice, which had approximately 70% less CD40 expression compared to that in the epididymal AT of WT mice (*Online Supplementary Figure S1A*). No effects on body weight were observed between the two genotypes (*Online Supplementary Figure S1B*).

### Adipocyte CD40 deficiency decreases hematopoiesis and increases T-cell activation

Adipocytes are abundantly present in hematopoietic and lymphoid organs where they interact closely with various cells, including immune cells. Here we investigated the effect of adipocyte CD40 deficiency on immune cell progenitors and immune cells in 22-week-old adult mice.

AdiCD40<sup>KO</sup> showed a decrease in Lin<sup>-</sup>Sca<sup>+</sup>cKit<sup>+</sup> (LSK) hematopoietic stem cells (HSC) and common lymphoid progenitor (CLP) cells in BM (Figure 1A, B, *Online Supplementary Figure S2A*, *Online Supplementary Figure, Gating strategies Bone Marrow*). The reduction in CLP resulted in a significant decrease in total BM B cells (Figure 1C, *Online Supplementary Figure S2B*). The total number of BM T cells did not differ between genotypes, but an increase in BM effector memory T cells (CD62L<sup>-</sup>CD44<sup>+</sup>) was observed, indicating a state of increased T-cell activation in the BM of adipocyte CD40-deficient mice (Figure 1D, *Online Supplementary Figure S2C*).

Histological analysis of the BM showed minor degenerative changes in the absence of adipocyte CD40, e.g., a trend for increased BM adiposity and a decrease in megakaryocytes (*Online Supplementary Figure S2D*).

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<sup>KO</sup> mice compared to WT littermates ( $P=0.23$ ) (*Online Supplementary Figure S2E*, *Online Supplementary Figure, Gating strategies Thymus*). Furthermore, the AdiCD40<sup>KO</sup> mice showed a reduction in thymic T-cell development, with development stagnating at the double-negative 2 stage, while further double-negative stages until the double-positive stage were decreased (Figure 1E, *Online Supplementary Figure S2F*). Although the amount of double-positive T cells was decreased, the output of single-positive CD4<sup>+</sup> and CD8<sup>+</sup> T cells was similar between genotypes (Figure 1F, *Online Supplementary Figure S2G*), indicating that negative selection on double-positive T cells is decreased in AdiCD40<sup>KO</sup> mice.

Glucocorticoids are closely related to thymocyte selection, with glucocorticoids opposing thymocyte negative selection.<sup>21</sup> Adipocyte CD40 deficiency did not result in structural changes to the adrenal glands (*Online Supplementary Figure S2H*), but AdiCD40<sup>KO</sup> mice did display increased plasma corticosterone levels (*Online Supplementary Figure S2H*), which may be responsible for the observed decrease in thymocyte negative selection.

### AdiCD40<sup>KO</sup> mice have altered immune cell composition in lymphoid organs

We further investigated immune cell composition in lymphoid organs of AdiCD40<sup>KO</sup> mice. We observed a decrease in B cells in spleen and lymph nodes, whereas the number of CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, and regulatory T cells was not affected (*Online Supplementary Figure S3A*). However, just as in the BM, there was an increase in memory T cells, whereas the naïve T-cell population decreased (*Online Supplementary Figure S3A, B*). An increase in T-cell activation could be confirmed *in vitro*, as CD4<sup>+</sup> and CD8<sup>+</sup> AdiCD40<sup>KO</sup> T cells stimulated for 72 h with CD3-CD28 antibody-coated beads showed increased IFN $\gamma$  and IL2 production (*Online Supplementary Figure S3C, D*). We did not observe changes in the myeloid cells in spleens of adipocyte CD40-deficient mice (*Online Supplementary Figure S3E*), and the BM myeloid progenitors also did not show major differences (*Online Supplementary Figure S3F*). These findings indicate that adipocyte CD40 deficiency has a direct impact on hematopoiesis and lymphopoiesis, as well as T-cell activation in adult mice.

### Aged adipocyte CD40-deficient mice have fewer lymphoid progenitors

Aging is associated with similar features of BM degeneration as observed in adult AdiCD40<sup>KO</sup> mice. We therefore hypothesized that the phenotype described above would be more prominent in aged AdiCD40<sup>KO</sup> mice. Indeed, in 52-week-old AdiCD40<sup>KO</sup> mice, we observed a significant increase in BM adiposity (Figure 2A), as well as absolute and relative decreases in LSK cells, including long-term and short-term stem cells, multipotent progenitors 1 (MPP1), MPP2, and MPP4 (Figure 2B). The most pronounced decrease was observed in the MPP4 population, associated with B- and T-cell development. Thus, aged AdiCD40<sup>KO</sup> mice had a decrease in CLP and late CLP (Lin<sup>-</sup>CD127<sup>+</sup>CD27<sup>low</sup>CD25<sup>low</sup>) populations (Figure 2C, *Online Supplementary Figure S4A*). Myeloid progenitors, derived from MMP3, did not show significant changes (*Online Supplementary Figure S4B*).

### Adipocyte CD40 deficiency results in a compensatory increase in bone marrow effector memory T cells

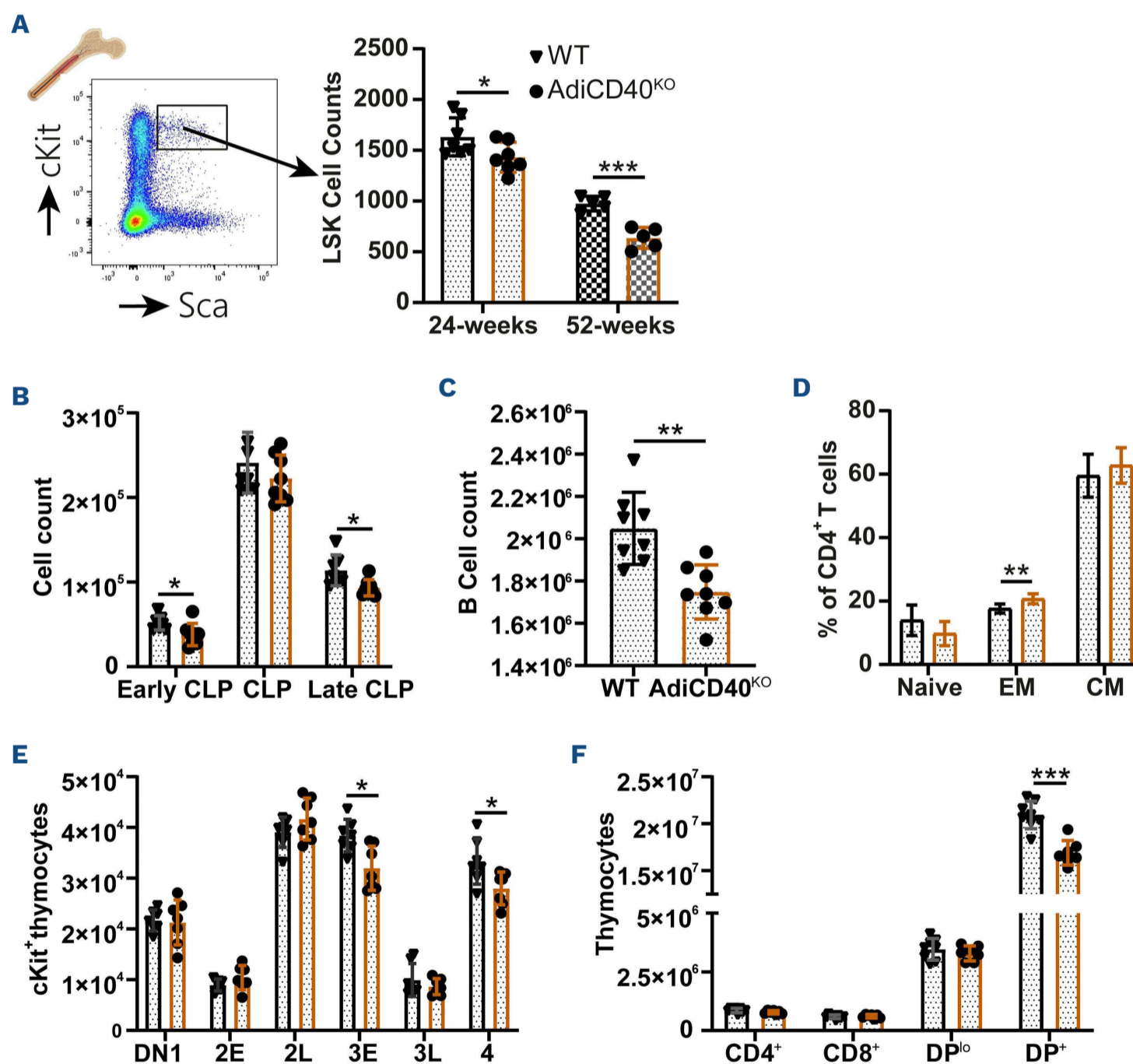
As most progenitor subclasses were reduced in the absence of adipocyte CD40, the number of BM CD45<sup>+</sup> cells decreased slightly (*Online Supplementary Figure S4C*). Remarkably, the total number of BM T cells was not affected in aged AdiCD40<sup>KO</sup> mice. Adipocyte CD40 deficiency caused a strong increase in CD4<sup>+</sup> and CD8<sup>+</sup> effector memory T cells (Figure 2D, *Online Supplementary Figure S4D*). 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.<sup>22</sup>

AdiCD40<sup>KO</sup> mice showed increased plasma corticosterone levels compared to WT mice (Figure 2E), and increased CXCR4 expression on T cells (Online Supplementary Figure S4E). This indicates that although T-cell development in BM is compromised when adipocyte CD40 is absent, effector memory T cells will be retrieved by the BM, thereby ensuring normal T-cell counts.

### Aged AdiCD40<sup>KO</sup> mice have a decrease in B cells in lymphoid organs and bone marrow

In line with observations in adult AdiCD40<sup>KO</sup> mice, we observed an increase of effector memory T cells in spleens of 52-week-old AdiCD40<sup>KO</sup> mice (Online Supplementary Figure

S4F). 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.<sup>23</sup> 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 AdiCD40<sup>KO</sup> mice (Online Supplementary Figure S4G). Furthermore, in the BM, ProB and PreB cells were decreased (Figure 2F, Online Supplementary Figure S4H), along with a significant decrease of IgG levels in BM interstitial fluid (Online Supplementary Figure S4I). These findings indicate either a decrease in hematopoietic output of, or a differentiation defect in, B cells from aged adipocyte CD40-deficient mice.



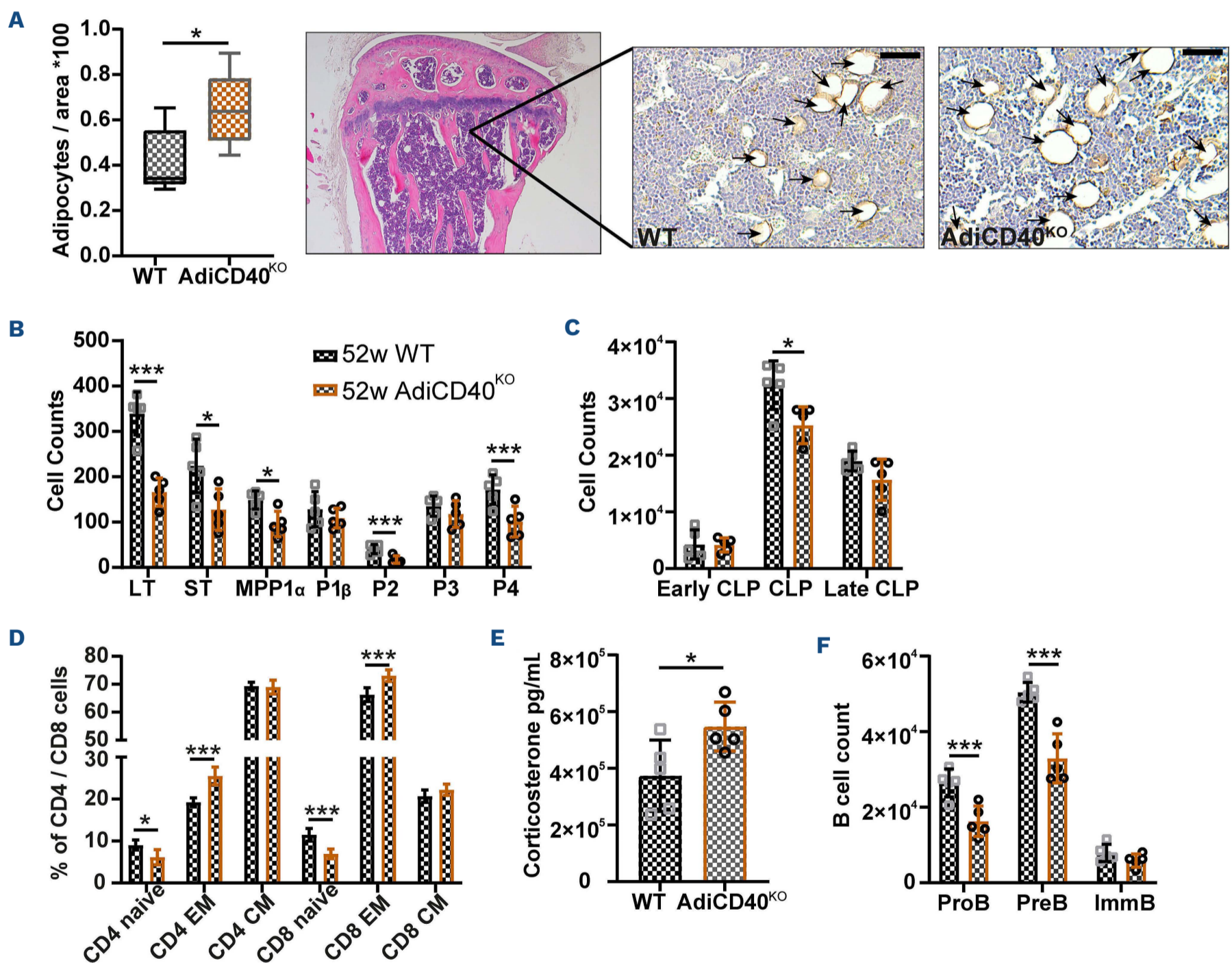
**Figure 1. Bone marrow composition and thymic selection are altered by adipocyte CD40 deficiency.** (A) Flow cytometric gating and analysis of one-tenth of bone marrow Lin<sup>-</sup>Sca<sup>+</sup>cKit<sup>+</sup> (LSK) cells of 22-week-old AdiCD40<sup>KO</sup> mice and wildtype littermates. (B) Total number of common lymphoid progenitors (CLP), Early CLP (Lin<sup>-</sup>CD135<sup>+</sup>CD127<sup>+</sup>), CLP, and Late CLP (Lin<sup>-</sup>CD127<sup>+</sup>CD27<sup>lo</sup>CD25<sup>lo</sup>). (C) Total number of B cells in bone marrow. (D) Activation status of CD4<sup>+</sup>, naïve (CD62L<sup>+</sup>CD44<sup>-</sup>), effector memory (CD62L<sup>-</sup>CD44<sup>+</sup>), and central memory (CD62L<sup>+</sup>CD44<sup>+</sup>) T cells in bone marrow. (E) Early thymocyte (cKit<sup>+</sup>) development of double-negative (CD25<sup>lo-hi</sup> CD44<sup>lo-hi</sup>) cells. (F) Selection of double-positive thymocytes (cKit<sup>+</sup>) into single-positive CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Data are shown as mean ± standard deviation of eight AdiCD40<sup>KO</sup> mice and eight wildtype littermates. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001. WT: wildtype; CLP: common lymphoid progenitor; EM: effector memory; CM: central memory; DN: double-negative; E: early; L: late; DP: double-positive.

### Adipocyte CD40 in cardiometabolic disease: atherosclerosis

To further investigate the role of adipocyte CD40 deficiency in hematopoiesis and lymphopoiesis in a chronic inflammatory disease, we backcrossed *AdiCD40<sup>KO</sup>* mice with *ApoE<sup>-/-</sup>* mice to obtain *AdipoQCre-CD40<sup>fl/fl</sup>-ApoE<sup>-/-</sup>* (*CD40<sup>AKO</sup>*) and *CD40<sup>fl/fl</sup>-ApoE<sup>-/-</sup>* (*ApoE<sup>-/-</sup>*) littermates to induce atherosclerosis. We found no major differences in body weight, plasma lipid levels including total cholesterol and very low-, low-, and high-density lipoprotein levels, or in total triglyceride levels between *CD40<sup>AKO</sup>* and *ApoE<sup>-/-</sup>* mice fed a 0.15% high cholesterol diet for 11 weeks (*Online Supplementary Figure S5*).

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

Under hypercholesterolemic conditions, *CD40<sup>AKO</sup>* mice exhibited an increase in both MPP3 (myeloid progenitors) and MPP4 (lymphoid progenitors) (Figure 3A), contrasting with the findings in our normocholesterolemic *AdiCD40<sup>KO</sup>* mice. Monocytosis was substantiated by an increase in downstream monocyte progenitor subsets such as common monocyte precursors and associated progenitors (Figure 3B, *Online Supplementary Figure S6A*). Previous studies have shown that hypercholesterolemia and/or ApoE deletion in humans and mice induce(s) BM monocytosis.<sup>24,25</sup>



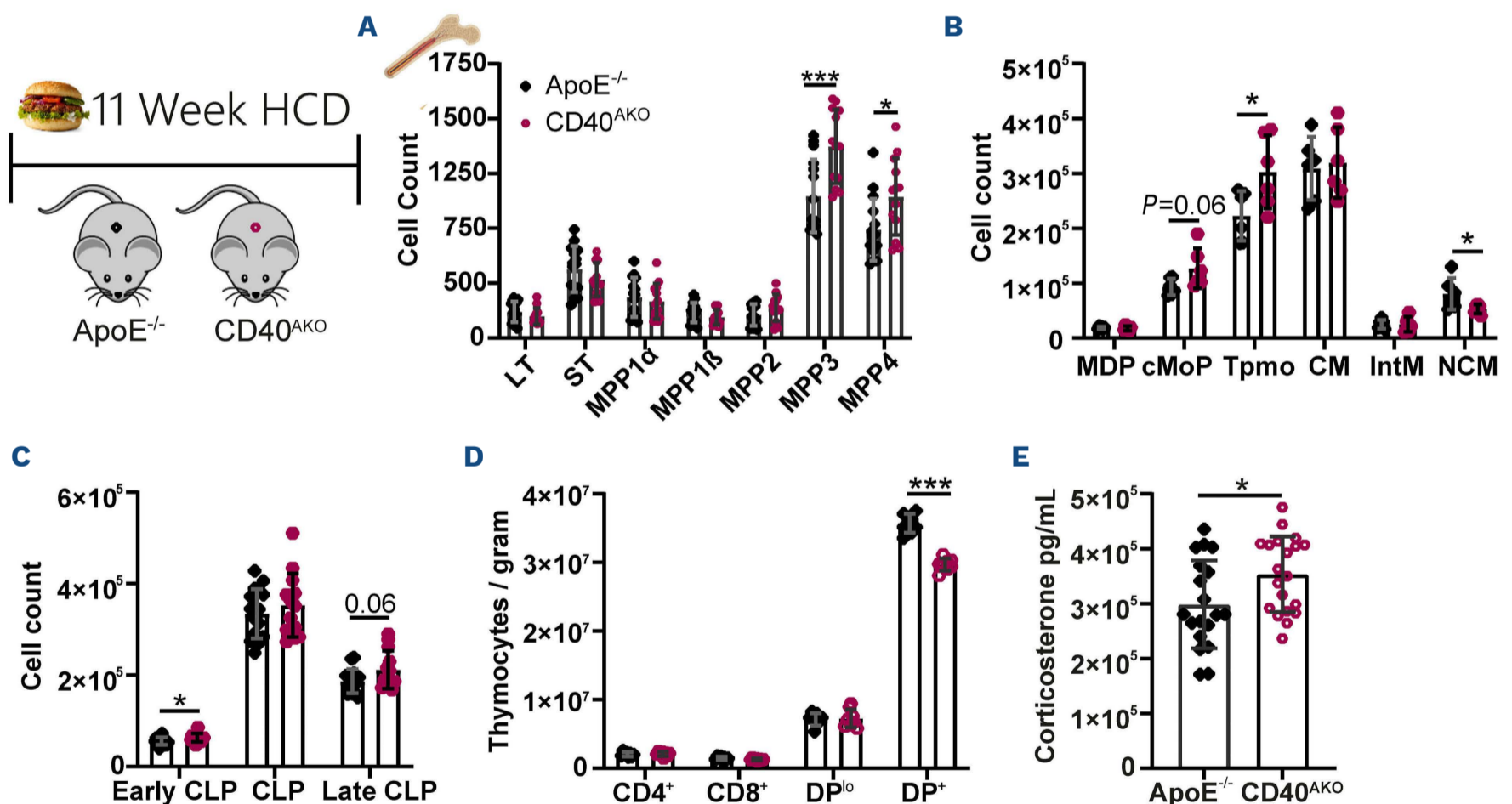
**Figure 2. Adipocyte CD40 deficiency has degenerative effects on the bone marrow.** (A) Quantification of adipocytes per area in the bone marrow of 52-week-old *AdiCD40<sup>KO</sup>* mice (n=5) and WT littermates (n=5), along with representative perilipin-1 staining of bone marrow adipocytes (scale bar = 25  $\mu$ m). (B) Flow cytometric analysis of one-tenth of the total number of long-term and short-term hematopoietic stem cells, and multipotent progenitors in bone marrow. (C) Total number of Early common lymphoid progenitors (CLP), CLP, and Late CLP. (D) Activation status of CD4<sup>+</sup> T cells in bone marrow. (E) Plasma corticosterone levels. (F) Flow cytometric analysis of B-cell maturation in bone marrow, indicating ProB, PreB, and immature B cells. Data are shown as mean  $\pm$  standard deviation for five *AdiCD40<sup>KO</sup>* mice and five WT littermates. \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.001. WT: wildtype; LT: long-term; ST: short-term; MPP: multipotent progenitors; CLP: common lymphoid progenitors; EM: effector memory T cells; CM: central memory T cells.

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, *Online Supplementary Figure S6B*). Furthermore, CD40<sup>AKO</sup> mice showed T-cell activation in the BM as well as systemically (*Online Supplementary Figure S6C-F*), similar to *AdiCD40<sup>KO</sup>* mice (Figure 1D). These data could be confirmed *in vitro* (*Online Supplementary Figure S6G*), and plasma levels of IFN $\gamma$  and TNF $\alpha$ , related to T-cell activation, were also increased (*Online Supplementary Figure S6H*).

The thymus of CD40<sup>AKO</sup> mice was seeded with an increased number of pre-thymocytes (cKit<sup>+</sup>) derived from BM CLP (*Online Supplementary Figure S6I*), while thymic development and selection were unaltered, leading to a similar output of single-positive T cells as that of ApoE<sup>-/-</sup> littermates (Figure 3D). This was associated with an increase in plasma corticosterone levels in CD40<sup>AKO</sup> 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 peripheral immune cell composition during hypercholesterolemia. Blood and spleen showed a similar number of CD45<sup>+</sup> leukocytes in CD40<sup>AKO</sup> and ApoE<sup>-/-</sup> littermates (*Online Supplementary Figure S7A*), but CD40<sup>AKO</sup> mice displayed an increase in monocytes (Figure 4A, *Online Supplementary Figure S7B*). *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, *Online Supplementary Figure S7C*). A significant decrease in B cells was observed in the spleen and circulation (Figure 4C, *Online Supplementary Figure S7D*). Similar to the observations in 22-week-old *AdiCD40<sup>KO</sup>* mice (*Online Supplementary Figure S4H*), CD40<sup>AKO</sup> 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<sup>AKO</sup> and ApoE<sup>-/-</sup> littermates (Figure 4E). These data



**Figure 3. Hypercholesterolemic adipocyte CD40-deficient mice have increased myelopoiesis and lymphopoiesis.** (A) Flow cytometric analyses of hematopoietic stem cells and one tenth of the total number of long-term and short-term hematopoietic stem cells, along with multipotent progenitors 1-4 in CD40<sup>AKO</sup> mice (n=15) and ApoE<sup>-/-</sup> littermates (n=15). (B) Bone marrow monocyte precursors, macrophage and dendritic cell progenitors, common monocyte precursors, transitional pre-monocytes, classical monocytes, intermediate monocytes, and non-classical monocytes in CD40<sup>AKO</sup> mice (n=6) and ApoE<sup>-/-</sup> littermates (n=6). (C) Early CLP, CLP, and Late CLP in bone marrow in CD40<sup>AKO</sup> mice (n=15) and ApoE<sup>-/-</sup> littermates (n=15). (D) Selection of double-positive thymocytes (cKit<sup>+</sup>) into single-positive CD4<sup>+</sup> and CD8<sup>+</sup> T cells: CD40<sup>AKO</sup> mice (n=8) and ApoE<sup>-/-</sup> littermates (n=8). (E) Plasma corticosterone levels in CD40<sup>AKO</sup> mice (n=20) and ApoE<sup>-/-</sup> littermates (n=19). Data are shown as mean  $\pm$  standard deviation. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . HCD: high cholesterol diet; LT: long-term hematopoietic stem cells; ST: short-term hematopoietic stem cells; MPP: multipotent progenitors; MDP: macrophage and dendritic cell progenitors, cMoP: common monocyte precursors; Tpmo: transitional premonocytes; CM: classical monocytes; IntM: intermediate monocyte; NCM: non-classical monocyte; CLP: common lymphoid progenitors; DP: double-positive.

show that adipocyte CD40 deficiency aggravates hypercholesterolemia-induced myelopoiesis and lymphopoiesis, while the opposite is shown for B-cell development.

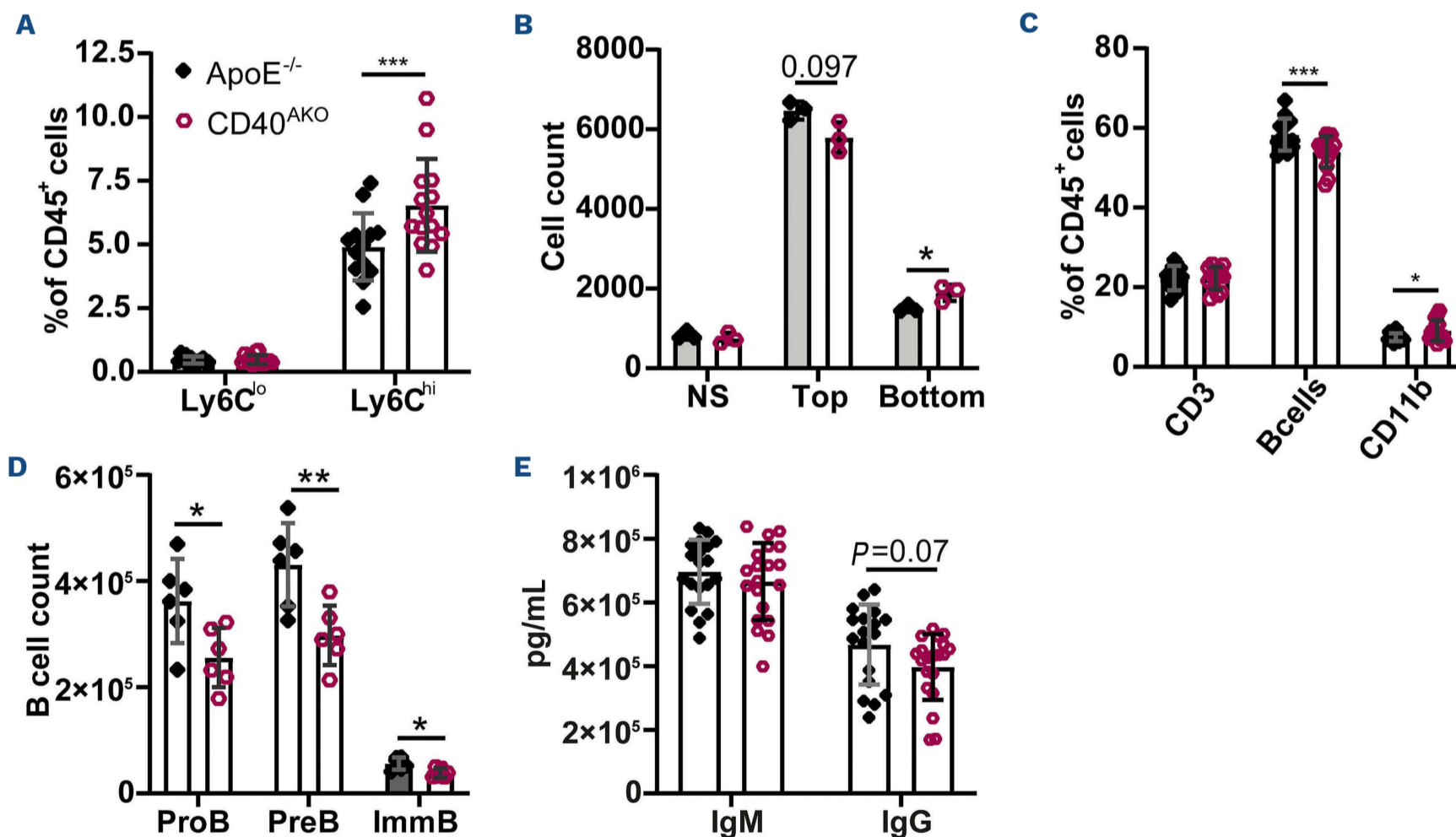
#### Adipocyte CD40 deficiency decreases atherosclerotic lesion size but induces necrotic core formation

Histological analysis of the aortic sinus showed mainly advanced lesions (fibrous cap atheromas) in both groups (*Online Supplementary Figure S8A*). The atherosclerotic plaques of CD40<sup>AKO</sup> mice were significantly smaller than those of ApoE<sup>-/-</sup> littermates (Figure 5A). Although atherosclerotic lesions were smaller in CD40<sup>AKO</sup> mice, the necrotic core size was significantly larger (Figure 5B). Concordant with the increase in necrotic core size, plaque macrophage content decreased (Figure 5C), along with a reduction in plaque T-cell content in CD40<sup>AKO</sup> mice (Figure 5D). However, the number of TUNEL<sup>+</sup> apoptotic cells, or apoptotic MAC3<sup>+</sup> macrophages was not significantly different in the absence of adipocyte CD40 (*Online Supplementary Figure S8B*), indicating efferocytosis was not affected in the plaques. Collagen and smooth muscle cell content did not differ (*Online Supplementary Figure S8C, D*).

Flow cytometric analysis revealed a decrease in total

CD45<sup>+</sup> lymphocytes in the aortic root and arch of CD40<sup>AKO</sup> mice (*Online Supplementary Figure S8E*). 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 (*Online Supplementary Figure S8F*). However, the Ly6C monocyte population showed an increase in Ly6C<sup>high</sup>/Ly6C<sup>low</sup> ratio, demonstrating a more activated monocyte profile in CD40<sup>AKO</sup> aortas (*Online Supplementary Figure S8G, H*). Additionally, CD3<sup>+</sup> 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<sup>AKO</sup> and ApoE<sup>-/-</sup> mice, as recent data indicated that cardiac/perivascular AT is associated with severity of cardiovascular disease.<sup>26</sup> Flow cytometric analysis of adipocyte CD40-deficient cardiac/perivascular AT showed an increase in macrophage content (*Online Supplementary Figure S8I*). 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<sup>AKO</sup> mice.



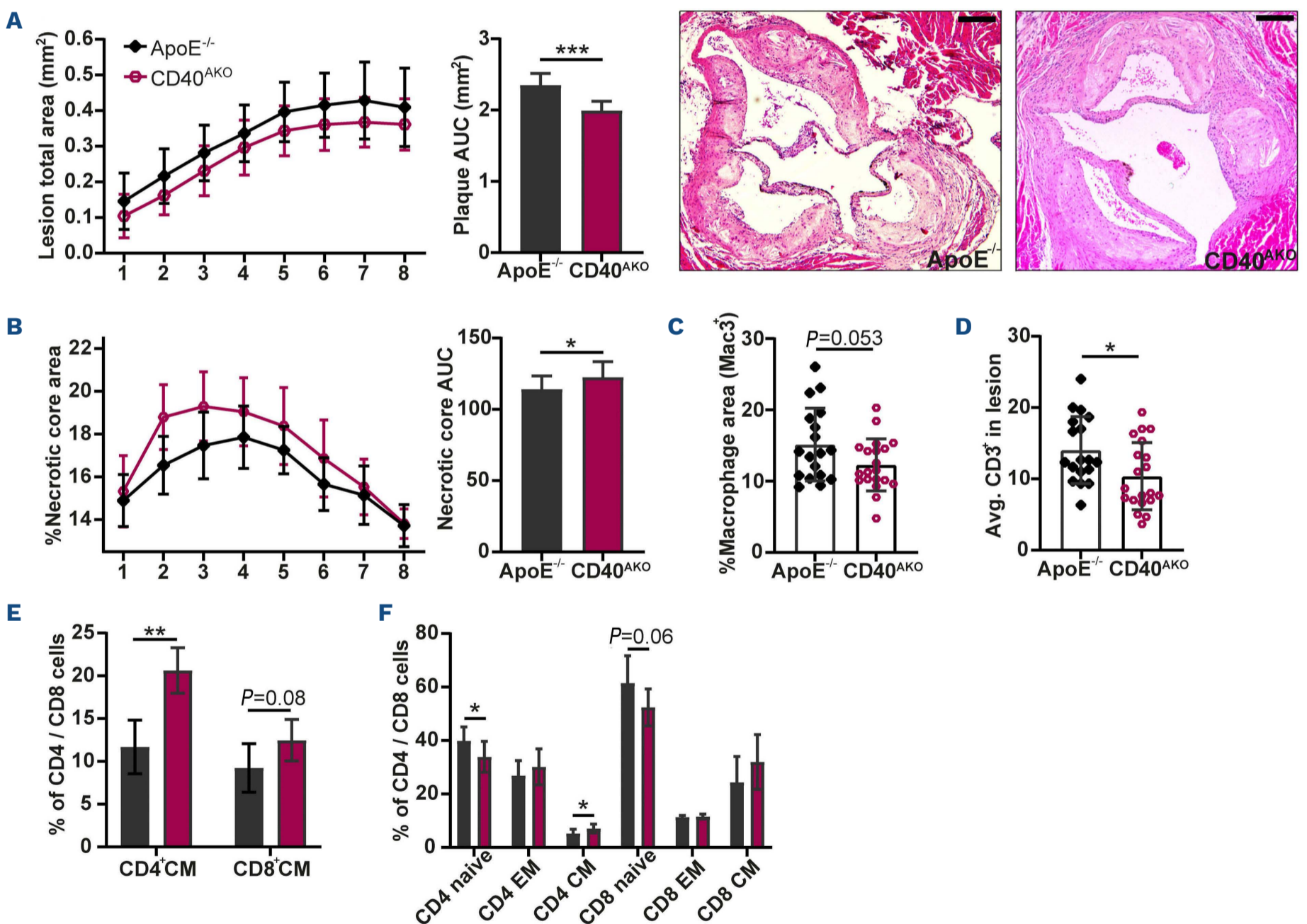
**Figure 4. Hypercholesterolemic adipocyte CD40-deficient mice have an altered immune cell composition.** (A) Flow cytometric analysis of Ly6C monocytes in spleens of CD40<sup>AKO</sup> mice (n=14) and ApoE<sup>-/-</sup> littermates (n=13). (B) Chemotactic transwell assay, analyzed by flow cytometry, of blood monocytes isolated from the top of the transwell, the bottom of the transwell without chemoattractant (NS), and from the bottom of the transwell with 10 ng/mL CCL2 (CD40<sup>AKO</sup> mice [n=3]; ApoE<sup>-/-</sup> littermates [n=3]). (C) Flow cytometric analysis of splenic CD3<sup>+</sup> T cells, CD19<sup>+</sup> B cells, and CD11b<sup>+</sup> innate immune cells (CD40<sup>AKO</sup> mice [n=14]; ApoE<sup>-/-</sup> littermates [n=13]). (D) B-cell progenitors in bone marrow (CD40<sup>AKO</sup> mice [n=6]; ApoE<sup>-/-</sup> littermates [n=6]). (E) Plasma IgM and IgG in CD40<sup>AKO</sup> mice (n=20) and ApoE<sup>-/-</sup> littermates (n=19). Data are shown as mean ± standard deviation. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001.



### Adipocyte CD40 in peripheral adipose tissue: obesity and 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 model by subjecting adipocyte CD40-deficient  $\text{AdiCD40}^{\text{KO}}$  mice and WT littermates to a standard-fat or high-fat diet for 15 weeks. All mice significantly gained weight during the period of the dietary intervention. However, adipocyte CD40 deficiency resulted in approximately 15% reduction in weight gain in mice fed the standard-fat or high-fat diet compared to the WT littermates (Figure 6A).  $\text{AdiCD40}^{\text{KO}}$  mice had slight reductions in epididymal AT and liver weights, and adipocyte size was similar between genotypes (Online Supplementary Figure S9A). After 12 weeks of dietary

intervention, glucose and insulin tolerance tests were performed.  $\text{AdiCD40}^{\text{KO}}$  mice and WT littermates fed the standard-fat diet remained glucose tolerant, and no differences were detected between genotypes. In contrast, mice fed the high-fat diet became glucose intolerant, but  $\text{AdiCD40}^{\text{KO}}$  mice exhibited improved glucose tolerance compared to their WT counterparts (Figure 6B, Online Supplementary Figure S9B). No differences were observed in plasma glucose and insulin levels (Online Supplementary Figure S9C, D).  $\text{AdiCD40}^{\text{KO}}$  mice had slightly reduced leptin and cholesterol levels, while plasma triglyceride levels were unaltered between genotypes (Online Supplementary Figure S9E-G). These data indicate that adipocyte CD40 deficiency diminishes manifestations of metabolic derangements in obese mice.

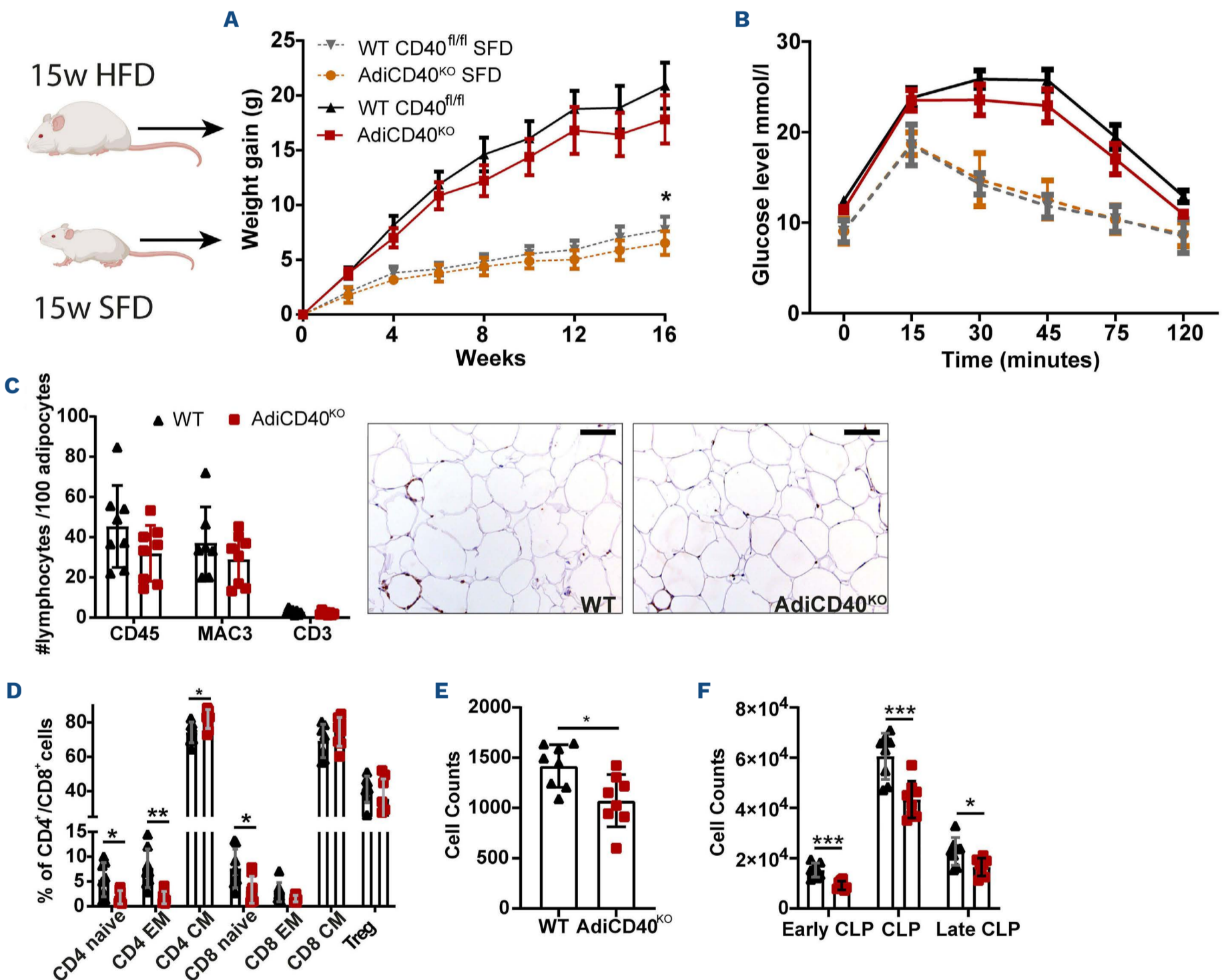


**Figure 5. Atherosclerotic lesion composition is altered and lesion size is decreased in  $\text{CD40}^{\text{AKO}}$  mice.** (A) Histological analysis of total lesion size of aortic roots, along with area under the curve measurements in  $\text{CD40}^{\text{AKO}}$  mice (n=20) and  $\text{ApoE}^{-/-}$  littermates (n=19), with representative images (scale bar = 100  $\mu\text{m}$ ). (B) Percentage total necrotic core area in lesions, along with area under the curve ( $\text{CD40}^{\text{AKO}}$  mice [n=10];  $\text{ApoE}^{-/-}$  littermates [n=19]). (C) Percentage macrophage (MAC3<sup>+</sup>) area in lesion. (D) Number of CD3<sup>+</sup> T cells per area in lesion. (E) Central memory CD4<sup>+</sup> and CD8<sup>+</sup> T cells in aortic root and aortic arch ( $\text{CD40}^{\text{AKO}}$  mice [n=6];  $\text{ApoE}^{-/-}$  littermates [n=6]). (F) Activation status of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in cardiac/perivascular adipose tissue ( $\text{CD40}^{\text{AKO}}$  mice [n=6];  $\text{ApoE}^{-/-}$  littermates [n=6]). Data are shown as mean  $\pm$  standard deviation. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . AUC: area under the curve; Avg: average; CM: central memory T cells; EM: effector memory T cells.

**Obese AdiCD40<sup>KO</sup> mice have fewer immune cells in adipose tissue**

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 groups that received the standard-fat diet. However, the epididymal AT of AdiCD40<sup>KO</sup> mice fed the high-fat diet contained slightly fewer CD45<sup>+</sup> leukocytes, MAC3<sup>+</sup> macrophages and crown-like structures (*Online Supplementary Figure S10A*), as well as CD3<sup>+</sup> T cells, compared to the epididymal AT of WT mice (Figure 6C). Flow

cytometric analysis of the stromal vascular fraction corroborated the slight decrease in CD45<sup>+</sup> cells in epididymal AT of AdiCD40<sup>KO</sup> mice fed the high-fat diet (*Online Supplementary Figure S10B*). Furthermore, just as in the aged AdiCD40<sup>KO</sup> mice and hypercholesterolemic CD40<sup>AKO</sup> mice, T-cell activation in epididymal AT of AdiCD40<sup>KO</sup> mice fed the high-fat diet increased. Both naïve and effector memory T cells decreased, while central memory T cells were increased (Figure 6D). We observed similar changes in blood, with a decrease in naïve T cells, and an increase in effector memory T cells (*Online Supplementary Figure S10C*). In accordance with the increase in circulating ef-



**Figure 6. Adipocyte CD40-deficient mice have decreased weight gain and more activated T cells.** (A) Weight gain in AdiCD40<sup>KO</sup> and wildtype mice fed a standard-fat diet (SFD, n=7) or a high-fat diet (HFD, n=8). (B) Glucose level and clearance over time, evaluated by a glucose tolerance test. (C) Histological quantification of total leukocytes (CD45<sup>+</sup>), macrophages (MAC3<sup>+</sup>) and T cells (CD3<sup>+</sup>), and representative images of epididymal adipose tissue of HFD-fed AdiCD40<sup>KO</sup> and wildtype mice (scale bar = 100 μm). (D) Flow cytometric analysis of activation status of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in epididymal adipose tissue. (E) One tenth of LSK cells in bone marrow of HFD-fed AdiCD40<sup>KO</sup> and wildtype mice. (F) Common lymphoid progenitors in bone marrow. Data are shown as mean ± standard deviation of AdiCD40<sup>KO</sup> mice fed the SFD (n=7) and HFD (n=8), and wildtype littermates fed the SFD (n=7) and HFD (n=8). \*P<0.05, \*\*P<0.01, \*\*\*P<0.001. w: week; HFD: high-fat diet; SFD: standard-fat diet; WT: wildtype; EM: effector memory T cells; CM: central memory T cells; Treg: regulatory T cells; CLP: common lymphoid progenitors.

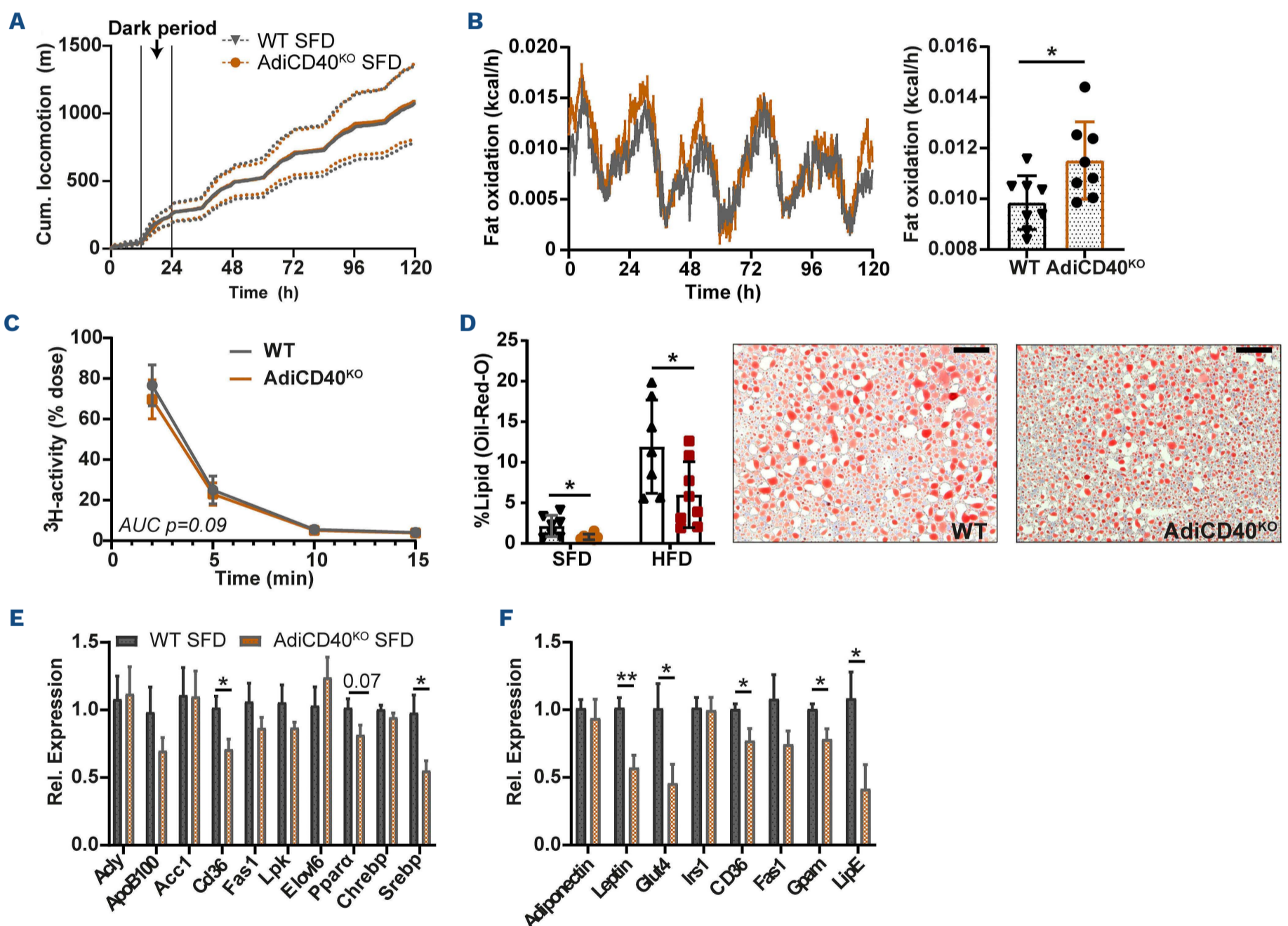
factor memory T cells, plasma of the *AdiCD40<sup>KO</sup>* mice showed increased IFN $\gamma$ , IL4, and IL17 levels (*Online Supplementary Figure S10D*). Interestingly, we did not observe significant changes in the thymus of *AdiCD40<sup>KO</sup>* mice fed the high-fat diet compared to WT littermates (*Online Supplementary Figure S10E*).

In the BM, we found that a high-fat diet exacerbated the effect of adipocyte CD40 deficiency on HSC, as LSK and CLP populations were decreased, while T-cell activation status increased (Figure 6E, F, *Online Supplementary Figure S10F, G*), as was observed in aged and atherosclerotic *AdiCD40<sup>KO</sup>* mice. In accordance with the findings in adult and atherosclerotic mice, obese mice have aggravated hematopoietic defects when adipocyte CD40 is deficient.

### Fat oxidation is increased in *AdiCD40<sup>KO</sup>* mice

As the phenotype of our mice is propagated by adipocytes, and adipocytes play a major role in whole-body metabolism, we wanted to explore whether the adipocyte CD40-deficiency phenotype affected metabolism, and thereby inflammation and hematopoiesis.

Therefore, 14-week-old mice that had been fed a standard-fat diet for 6 weeks were individually housed in fully automated metabolic cages; at that time body weights of the *AdiCD40<sup>KO</sup>* mice and their WT littermates were still comparable. Voluntary locomotor activity and food intake were not affected by adipocyte CD40 deficiency (Figure 7A, *Online Supplementary Figure S11A*). Energy expenditure, as estimated from VO $_2$  and VCO $_2$  and normalized to fat-free mass,<sup>27</sup> was increased during both



**Figure 7. Fat oxidation is increased in *AdiCD40<sup>KO</sup>* mice.** (A) Cumulative voluntary locomotion over time, as observed in a metabolic cage, for *AdiCD40<sup>KO</sup>* mice (n=8) and wildtype mice (n=8) fed a standard-fat diet (SFD). (B) Indirect calorimetry measurement of total fat oxidation (kcal/h) rate and quantification during the light period. (C) Clearance of injected tri[<sup>3</sup>H]oleate over time as determined in blood (*AdiCD40<sup>KO</sup>* mice [n=8]; wildtype mice [n=8]). (D) Oil-Red-O staining for lipids in livers of mice fed the SFD (n=8) or the high-fat diet (n=8), with representative images from the latter. (E) mRNA expression of genes involved in metabolism, liver. (F) Expression of genes involved in epididymal adipose tissue metabolism. Data are shown as mean  $\pm$  standard deviation for *AdiCD40<sup>KO</sup>* mice (n=8) and wildtype littermates (n=8) fed the SFD. \* $P<0.05$ , \*\* $P<0.01$ . Cum: cumulative; WT: wildtype; SFD: standard-fat diet; HFD: high-fat diet; Rel.: relative.

the (resting) light period and (active) dark period in *AdiCD40<sup>KO</sup>* mice (*Online Supplementary Figure S11B*), although the increase was not statistically significant. More specifically, fat oxidation was found to be increased during the light period (Figure 7B, C), resulting in a decreased respiratory quotient (*Online Supplementary Figure S11C-E*).

After 8 weeks of the standard-fat diet, mice were injected with [<sup>14</sup>C]deoxyglucose and lipoprotein-like particles containing glycerol tri[<sup>3</sup>H]oleate. Plasma clearance of the glycerol tri[<sup>3</sup>H]oleate tracer tended to be accelerated in *AdiCD40<sup>KO</sup>* mice, although this effect could not be related to enhanced [<sup>3</sup>H]oleate uptake by a certain metabolic organ (Figure 7C, *Online Supplementary Figure S11F*). Plasma clearance of [<sup>14</sup>C]deoxyglucose was comparable between genotypes, although the liver had significantly more uptake of [<sup>14</sup>C]deoxyglucose particles (*Online Supplementary Figure S11G, H*). These data indicate some changes in the metabolic profile of AT and liver in adipocyte CD40-deficient mice.

Furthermore, livers of *AdiCD40<sup>KO</sup>* mice showed decreased levels of steatosis, which we had previously also observed in *AdiCD40<sup>KO</sup>* mice fed the high-fat diet (Figure 7D). Transcriptome analysis of livers from *AdiCD40<sup>KO</sup>* mice fed the standard-fat diet showed changes in cholesterol metabolism genes, including *Srebp* and *Ppara*, 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 to the liver. Indeed, transcriptome analysis of the AT from *AdiCD40<sup>KO</sup>* mice fed the standard-fat diet revealed decreased expression of glucose (*Glut4*) and *Cd36* receptors, along with a decrease in the *LipE* gene, which hydrolyzes stored triglycerides to free fatty acids (Figure 7F, *Online Supplementary Figure S11I*). These data indicate that adipocyte CD40 deficiency improves fatty acid turnover in AT and the 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 decreased plaque burden, but lesions had enlarged necrotic cores and contained more activated T cells. During diet-induced obesity, deficiency of adipocyte CD40 resulted in less weight gain, improved insulin sensitivity, and increased fat oxidation. Additionally, total lymphocytes in AT decreased, but there was an increase in the number of activated T cells.

## Discussion

With age, people become more vulnerable to the development of diseases.<sup>28</sup> This can be attributed, in part, to “inflammaging”. Metabolic inflammation brought on by nutrient excess and over-nutrition accelerates disease progression.<sup>29</sup> “Inflammaging” impairs hematopoiesis due to disruption of BM niches, limiting HSC survival, self-renewal, and differentiation.<sup>30</sup> Adipocytes have been found to play a major role in this process, as adipocytes accumulate and physically disrupt BM niches.<sup>7</sup> Furthermore, adipogenesis of the thymus aggravates thymic involution, thereby reducing the immune system’s ability to grow the T-cell repertoire.<sup>31,32</sup> 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 hematopoiesis 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.<sup>33</sup> We and others have shown that deletion of CD40 or CD40L on different types of cells has different effects on the progression of chronic inflammatory diseases.<sup>4,11-18</sup> Genetic studies in both patients and mice have shown that CD40L-CD40 signaling might affect BM hematopoiesis and, thus, inflammatory disease progression.<sup>34</sup> It was found that the interaction of CD40L with CD40 on HSC induces CD40-TNF receptor associated factor (TRAF)6 signaling, which activates NFκB in HSC.<sup>34</sup> This closely links CD40-CD40L with HSC and BM niche stability, which the data on adipocyte CD40 in the current study corroborate.

Adipocytes are not only a depot for lipid storage and producers of satiety hormones, but also interact directly with immune cells.<sup>9,35</sup> Leptin secreted by adipocytes induces IFNγ secretion by T cells.<sup>10</sup> IFNγ upregulates expression of major histocompatibility complex II on adipocytes, thereby enhancing adipocyte-T-cell interactions and activation.<sup>10</sup> Activation of adipocyte CD40 by CD40L increases the production of pro-inflammatory cytokines, upregulates CD40 expression, and increases adipocyte lipolysis.<sup>9,35,36</sup> In obese patients, adipocyte CD40 mRNA levels are positively correlated with body mass index, as well as gene expression of leptin and IL6.<sup>35</sup> Deficiency of adipocyte CD40 could therefore improve metabolic function and inflammation in AT, as we indeed observed in AT of adipocyte CD40-deficient mice.

Another observation that we made in adipocyte CD40-deficient mice was 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 retaining T cells in the BM, which was initiated by an increased release of glucocorticoids by

the adrenal glands.<sup>22,37</sup> Dietary restriction paradoxically increased adipogenesis inside the BM, while white AT deposits were decreased.<sup>37</sup> Interestingly, when BM adipocytes were depleted in Adipoq-CreERT2×Rosa26-DTA mice, memory T cells could no longer be retained in the BM.<sup>37</sup> It was suggested that BM adipocytes are crucial for memory T-cell maintenance and survival through the supply of long-chain fatty acids.<sup>38</sup> These data underscore our findings, as we found increased glucocorticoid levels and more (CXCR4<sup>+</sup>) effector T cells in BM and lymphoid organs, probably triggered by decreased availability of nutrients in CD40-deficient BM adipocytes.

It has been reported that BM adipocytes have distinct lipid metabolism compared to white AT, with BM adipocytes being more cholesterol-oriented,<sup>39</sup> which is crucial for HSC metabolism.<sup>40</sup> Furthermore, in BM adipocytes genes related to lipolysis are strongly reduced,<sup>39</sup> while the CD40-CD40L interaction is a strong inducer of lipolysis in white adipocytes.<sup>9,35,36</sup> 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<sup>-/-</sup> mice, we found a decrease in atherosclerotic 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.<sup>41</sup> Pro-inflammatory CD8<sup>+</sup> T cells have been found to promote the development of a vulnerable plaque, as antibody-mediated depletion of CD8<sup>+</sup> T cells in ApoE<sup>-/-</sup> mice reduces lipid and macrophage accumulation, apoptosis, and necrotic core content.<sup>42,43</sup> Furthermore, cytokines produced by activated CD4<sup>+</sup> T cells can activate macrophage lipid uptake and apoptosis in the atherosclerotic lesion.<sup>41</sup> Concomitantly, activated CD4<sup>+</sup> T cells in blood of patients with coronary artery disease are directly correlated with unstable lesion phenotypes.<sup>44</sup> 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 the progression of chronic inflammatory diseases. 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 and insulin sensitivity, thereby decreasing weight gain. In addition, adipose CD40 has a key regulatory role in BM homeostasis. Its absence affects BM cell composition and thereby hematopoiesis along with lymphopoiesis, resulting in increased T-cell activation, in models of age-related cardiometabolic disease.

### Disclosures

*No conflicts of interest to disclose.*

### Contributions

*MER, SABMA, CW, DA, SK, and EL conceived the study. MER, SABMA, CW, DA, PCNR, SK, and EL contributed to the design and implementation of the research. MER, MdT, and LAB performed experiments with the aged mice. MER, KP, LAB, CMvT, MdT, and LAB performed experiments with the hypercholesterolemic mice. MER, SABMA, MdT, LB, and SK performed experiments with the diet-induced obesity mice. MER, LAB, WGV, CMvT, and MJJG performed experiments and analysis for the revised manuscript. MER and EL wrote the paper.*

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### Data-sharing statement

*The data will be made available within 3 months from initial request through correspondence with the corresponding author.*

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