Monocyte-to-macrophage differentiation, which can be initiated by physiological or atherogenic factors, is a pivotal process in atherogenesis, a disorder in which monocytes adhere to endothelial cells and subsequently migrate into the subendothelial spaces, where they differentiate into macrophages and macrophage-derived foam cells and cause atherosclerotic lesions. However, the monocyte-differentiation signaling pathways that are activated by atherogenic factors are poorly defined. Here we report that the AMP-activated protein kinase $\alpha_1$ (AMPK$\alpha_1$) in monocytes promotes atherosclerosis by increasing monocyte differentiation and survival. Exposure of monocytes to oxidized low-density lipoprotein, $\gamma$-ketocaproate, phorbol 12-myristate 13-acetate, or macrophage colony-stimulated factor (M-CSF) significantly activated AMPK and promoted monocyte-to-macrophage differentiation. M-CSF-activated AMPK is via M-CSF receptor-dependent reactive oxygen species production. Consistently, genetic deletion of AMPK$\alpha_1$ or pharmacological inhibition of AMPK blunted monocyte-to-macrophage differentiation and promoted monocyte/macrophage apoptosis. Compared with apolipoprotein E knock-out (ApoE$^{-/-}$) mice, which show impaired clearing of plasma lipoproteins and spontaneously develop atherosclerosis, ApoE$^{+/+}$/AMPK$\alpha_1^{-/-}$ mice showed reduced sizes of atherosclerotic lesions and smaller numbers of macrophages in the lesions. Furthermore, aortic lesions were decreased in ApoE$^{-/-}$ mice transplanted with ApoE$^{-/-}$/AMPK$\alpha_1^{-/-}$ bone marrow and in myeloid-specific AMPK$\alpha_1$-deficient ApoE$^{-/-}$ mice. Finally, rapamycin treatment, which abolished delayed monocyte differentiation in ApoE$^{-/-}$/AMPK$\alpha_1^{-/-}$ mice, lost its atherosclerosis-lowering effects in these mice. Mechanistically, we found that AMPK$\alpha_1$ regulates FoxO3-dependent expression of both LC3 and ULK1, which are two important autophagy-related markers. Rapamycin treatment increased FoxO3 activity as well as LC3 and ULK1 expressions in macrophages from AMPK$\alpha_1^{-/-}$ mice. Our results reveal that AMPK$\alpha_1$ deficiency impairs autophagy-mediated monocyte differentiation and decreases monocyte/macrophage survival, which attenuates atherosclerosis in ApoE$^{+/+}$ mice in vivo.

Peripheral monocytes circulate in the blood for 24–48 h and undergo spontaneous apoptosis without appropriate stimulation (1–3). Atherogenic factors promote the adherence of monocytes to endothelial cells and their subsequent migration into the subendothelial spaces, where they are differentiated and transformed into macrophages and macrophage-derived foam cells (4, 5). Thus, monocyte-to-macrophage differentiation is a pivotal process in atherogenesis (6–8). Several factors, including oxidized low-density lipoprotein (ox-LDL)$^3$ particles, macrophage colony-stimulating factor (M-CSF), or granulocyte-macrophage colony-stimulating factor, play essential roles in this process (9–11). In mice lacking both M-CSF and apolipoprotein E (apoE), atherosclerotic lesions are smaller than those in apoE-deficient mice (ApoE$^{-/-}$), probably due to the nearly complete lack of monocytes in the peripheral blood and impairments in monocyte-to-macrophage differentiation (7, 8). M-CSF or ox-LDL has been known to activate phosphoinositide-3 kinase and mitogen-activated protein kinase pathways during differentiation (12, 13). Recent studies also show that the induction of autophagy prevents monocytes from apoptosis and is critical for M-CSF-induced monocyte-to-macrophage differentiation (14, 15). However, the precise molecular mechanisms by which they induce monocyte survival and differentiation are not fully understood.

AMP-activated protein kinase (AMPK) is an evolutionarily conserved serine/threonine kinase that consists of one catalytic subunit ($\alpha$) and two regulatory subunits ($\beta$ and $\gamma$) (16, 17). There are two $\alpha$ isoforms (AMPK$\alpha_1$ and $\alpha_2$), which are differentially expressed in different tissues. The primary function of AMPK is to act as the energy and redox sensor that is involved in metabolic regulation and insulin sensitivity (16, 17). Recent evidence also indicates novel roles for AMPK in the pathogen-
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thesis of cardiovascular diseases. AMPKα2 deletion increases atherosclerosis in ApoE−/− mice, probably via enhanced oxidative stress and endoplasmic reticulum stress (18). A recent study shows that AMPK activators metformin and AICAR significantly attenuated PMA-induced monocyte-to-macrophage differentiation in THP-1 cell and proinflammatory cytokine production (19). However, whether the effects of metformin or AICAR are AMPK-dependent is not clear because compound C, an inhibitor of AMPK, was ineffective in promoting monocyte-to-macrophage differentiation in the absence of PMA (19). The aim of the present study was to define the roles of AMPKα1, the predominant isoform expressed by monocytes/macrophages, in monocyte-to-macrophage differentiation and atherosclerosis. Our findings have revealed that the genetic deletion of AMPKα1 suppressed autophagy and autophagy-mediated monocyte differentiation, resulting in increased monocyotic cell death, all of which contributed to the reduction of atherosclerotic lesions in vivo.

Results

AMPKα1 inhibition impairs M-CSF- or ox-LDL-induced monocyte-to-macrophage differentiation in vitro

First, we detected the effects of AMPK inhibition on M-CSF-induced differentiation in human monocytes in vitro. As shown in Fig. 1A, M-CSF significantly increased CD71 expression, an indicator of monocyte-to-macrophage differentiation, whereas it decreased monocyte marker CD14 expression after 1 and 3 days of treatment. However, AMPK inhibition by compound C significantly suppressed M-CSF-induced CD71 expression and maintained CD14 expression at a higher level.

To examine whether AMPK is involved in ox-LDL-induced monocyte differentiation, THP-1 cells were treated with phorbol 12-myristate 13-acetate (PMA), ox-LDL, or 7-KC with or without AMPK inhibitor compound C. Ox-LDL or 7-KC significantly enhanced PMA-induced expressions of CD36 and CD11b, which are macrophage-differentiation markers. Meanwhile, compound C dramatically blocked the effects of ox-LDL and 7-KC on CD36 and CD11b expressions (Fig. 1, B and C).

AMPKα1 deletion slows down monocyte-to-macrophage differentiation in vivo

Thioglycollate broth injection into the peritoneal cavity causes the recruitment of circulating monocytes to the peritoneum, where they transform into tissue macrophages within 4 days (20). This approach has been used to study monocyte differentiation in vivo (15). To detect whether or not AMPKα1 deletion affects monocyte-to-macrophage differentiation in vivo, peritoneal Ly6C+hiF4/80lo monocytes and Ly6C+hiF4/80hi macrophages in CD11b−/−CD90−/−B220−/−CD49b−/−NK1.1−/−Ly6-G−/−mononuclear cells (21) were sorted 1–4 days after thioglycollate administration. As shown in Fig. 1D, 1 day after thioglycollate injection, 60–70% of cells in the peritoneal cavity were Ly6C+hiF4/80lo mature macrophages, which are resident macrophages in the peritoneal cavity. The percentages of Ly6C+hiF4/80lo monocytes and Ly6C+hiF4/80hi macrophages at day 1 were comparable in WT and AMPKα1−/− mice. However, the percentages of Ly6C+hiF4/80lo monocytes at days 2 and day 3 were significantly higher, whereas the percentage of Ly6C+hiF4/80hi macrophages from day 2 to day 4 in the peritoneum of AMPKα1−/− mice were significantly lower than their WT counterparts (Fig. 1D).

To establish whether AMPK slows down monocyte-to-macrophage differentiation in athrogenic animal models, thioglycollate was injected into ApoE−/− or ApoE−/−/AMPKα1−/− mice. As depicted in Fig. 1E, ApoE−/−/AMPKα1−/− mice also exhibit higher percentages of Ly6C+hiF4/80lo monocytes and lower percentages of Ly6C+hiF4/80hi macrophages than ApoE−/− mice during monocyte differentiation, although the total numbers of Ly6C+hiF4/80lo monocytes at day 3 were similar between the two groups (3.5 × 106 in ApoE−/− mice versus 3.0 × 106 in ApoE−/−/AMPKα1−/− mice). Taken together, these results suggest that AMPKα1 is important for thioglycollate-induced monocyte-to-macrophage differentiation.

AMPK is activated during monocyte differentiation

To test whether AMPK is activated during monocyte differentiation, phosphorylation of AMPK at threonine 172 was detected in bone marrow cells treated with M-CSF. As shown in Fig. 1, F and G, M-CSF significantly increased AMPK phosphorylation in both peripheral blood monocytes and isolated peritoneal monocytes/macrophages from ApoE−/− mice. M-CSF also largely enhanced AMPK phosphorylation in human monocytes (Fig. 1H) and AMPK activity in THP-1 cells (Fig. 1I). In addition, PMA, ox-LDL, and 7-KC all significantly increased AMPK phosphorylation in THP-1 cells, and 7-KC further increased PMA-induced AMPK phosphorylation (Fig. 1I).

M-CSF activates AMPK via M-CSF receptor-mediated reactive oxygen species (ROS) production

To explore whether M-CSF promoted AMPK activation through its receptor CD115, THP-1 cells were transfected with CD115 siRNA for 48 h. As shown in Fig. 1K, knockdown of CD115 significantly inhibited M-CSF-induced AMPK activation, implying that AMPK activation by M-CSF is CD115-dependent. It has been known that M-CSF stimulation results in the production of ROS (22, 23). We confirmed that ROS levels were increased in M-CSF-treated peritoneal monocytes/macrophages (Fig. 1L) and THP1 cells (Fig. 1M). Because ROS signaling is important in AMPK activation under various conditions (24), we further tested whether M-CSF activates AMPK via ROS production. M-CSF-treated peritoneal monocytes were incubated with or without ROS scavenger, 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPOL). As shown in Fig. 1N, TEMPOL significantly blocked the activation of AMPK induced by M-CSF.

AMPKα1 deletion increases cell apoptosis during monocyte-to-macrophage differentiation

Peripheral blood monocytes undergo apoptosis in the absence of appropriate differentiation stimuli (1–3). Therefore, impaired monocyte-to-macrophage differentiation increases cell apoptosis. We assessed whether AMPKα1 deletion accelerates cellular apoptosis during monocyte-to-macrophage differentiation. Peritoneal monocytes/macrophages were isolated...
from ApoE\(^{-/-}\) and ApoE\(^{-/-}/\text{AMPKa}1^{-/-}\) mice at 1 day after thioglycollate injection and stained with Annexin V and propidium iodide. The number of apoptotic cells markedly increased in ApoE\(^{-/-}/\text{AMPKa}1^{-/-}\) mice compared with that in ApoE\(^{-/-}\) mice (Fig. 2A). Similar results were observed in peritoneal monocytes/macrophages that were isolated from WT and AMPKa1\(^{-/-}\) mice (Fig. 2B). The numbers of TUNEL-positive macrophages significantly increased in ApoE\(^{-/-}/\text{AMPKa}1^{-/-}\) mice compared with that in ApoE\(^{-/-}\) mice (Fig. 2A).
AMPKα1−/− mice at day 3 after thioglycollate injection, thus further confirming macrophage apoptosis (Fig. 2C). The levels of cleaved poly(ADP-ribose) polymerase (PARP) and cleaved caspase-3 were also largely increased in both peritoneal and bone marrow-derived macrophages in ApoE−/−/AMPKα1−/− mice (Fig. 2D and E).

To confirm that AMPK inhibition increases monocyte/macrophage apoptosis during differentiation in humans, monocytes were isolated from human peripheral blood and incubated with compound C (5–10 μM) in the presence of 40 ng/ml M-CSF for 1 or 3 days. As shown in Fig. 2F, inhibition of AMPK by compound C dramatically increased cleaved caspase 3 expression. In contrast, overexpression of AMPKα1 in macrophages significantly inhibited 7-KC-, 25-OH cholesterol-, or staurosporine-induced apoptosis in peritoneal macrophages (Fig. 2G).

To investigate whether AMPKα1 deletion impaired monocyte differentiation and whether increased apoptosis has impacts on macrophage numbers, peritoneal cells were isolated and counted 1–4 days after thioglycollate injection. As shown in Fig. 2H, peritoneal cell numbers in ApoE−/−/AMPKα1−/− mice are dramatically decreased at days 3 and 4 compared with those in ApoE−/− mice. Similar results were observed in AMPKα1−/− and WT mice injected with thioglycollate. (Fig. 2I).

**Deletion of AMPKα1 does not affect the functions of mature macrophages**

Next, we determined whether AMPKα1 deletion alters the functions of macrophages. The phagocytic ability of bone marrow-derived macrophages was detected by measuring the uptake of pHrodo red Escherichia coli bioparticles. As shown in Fig. 3A, the numbers of internalized bioparticles by bone marrow-derived macrophages were similar between ApoE−/−/AMPKα1−/− mice and ApoE−/− mice.

To determine whether AMPKα1 modulates chemotactic activity, bone marrow-derived macrophages were used to examine monocyte chemotactic protein-1 (MCP-1)-induced cell migration. ApoE−/−/AMPKα1−/− and ApoE−/− macrophages exhibited similar chemotactic responses to MCP-1 (Fig. 3B). We further detected Dil-ox-LDL binding of macrophages. After incubation with Dil-ox-LDL for 1 h, both peritoneal and bone marrow-derived macrophages from ApoE−/−/AMPKα1−/− mice displayed similar ox-LDL-binding capability compared with macrophages from ApoE−/− mice (Fig. 3C, D). To further determine the effects of AMPKα1 on foam cell formation, peritoneal macrophages were loaded with 50 μg/ml ox-LDL for 48 h and then stained with Oil Red O. Macrophages from ApoE−/−/AMPKα1−/− and ApoE−/− mice exhibited similar intensity of Oil Red O staining (Fig. 3E).

**AMPKα1 deletion inhibits autophagy during monocyte differentiation**

Recent studies have suggested that autophagy is required for monocyte-to-macrophage differentiation and cell survival (14, 15). Meanwhile, increasing evidence supports the role of AMPK in the regulation of autophagy, especially during the autophagosome elongation stage (25, 26). To explore the effect of AMPK on autophagy during monocyte differentiation, we tracked the conversion of microtubule-associated protein light chain 3 (LC3)-I to LC3-II, which is used as an indicator of autophagic activity. As shown in Fig. 4A, PMA, ox-LDL, and 7-KC increased LC3-II expression in THP-1 cells, and ox-LDL could further increase PMA-induced LC3-II expression. We further detected LC3 expression during different times of monocyte differentiation. As shown in Fig. 4B, LC3-1/II expressions are dramatically increased during M-CSF-induced differentiation in ApoE−/− cells, whereas both LC3-I and LC3-II were largely decreased in ApoE−/−/AMPKα1−/− macrophages. When peritoneal macrophages were collected 3 days after thioglycollate injection, both LC3–1 and LC3–II expression levels were also significantly reduced in ApoE−/−/AMPKα1−/− mice compared with those in ApoE−/− mice (Fig. 4C).

We further transfected the green fluorescent protein (GFP)-LC3 adenovirus...
Figure 2. Genetic AMPKα1 deletion increases apoptosis in monocyte-to-macrophage differentiation. A, Annexin V-positive peritoneal cells from ApoE<sup>−/−</sup> and ApoE<sup>−/−</sup>/AMPKα<sup>−/−</sup> mice were isolated 1 day after thioglycollate injection. #, p < 0.05 versus ApoE<sup>−/−</sup> mice (n = 3). B, Annexin V-positive peritoneal cells were detected 1 day after thioglycollate injection in WT and AMPKα<sup>−/−</sup> mice. #, p < 0.05 versus WT mice (n = 4). C, peritoneal macrophages were isolated on day 3 after the injection of thioglycollate in ApoE<sup>−/−</sup> and ApoE<sup>−/−</sup>/AMPKα<sup>−/−</sup> mice. Cells were fixed and incubated with TUNEL solution and DAPI. Five different fields for each 35-mm culture plate were randomly selected to count the percentage of TUNEL-positive cells. #, p < 0.05 versus ApoE<sup>−/−</sup> mice. Scale bar, 50 μm (n = 3–5). D, cleaved PARP (C-PARP) and cleaved caspase-3 (C-Casp3) expression levels were detected by Western blotting in bone marrow-derived macrophages from ApoE<sup>−/−</sup> and ApoE<sup>−/−</sup>/AMPKα<sup>−/−</sup> mice. #, p < 0.01 versus ApoE<sup>−/−</sup> mice; *, p < 0.05 versus ApoE<sup>−/−</sup> mice. Data are representative of three independent experiments. E, cleaved PARP and cleaved caspase-3 were detected by Western blotting in peritoneal macrophages from ApoE<sup>−/−</sup> and ApoE<sup>−/−</sup>/AMPKα<sup>−/−</sup> mice. #, p < 0.01 versus ApoE<sup>−/−</sup> mice (n = 6). F, cleaved caspase-3 was detected by Western blotting in human monocytes that were treated with different concentrations of compound C for 1 and 3 days. #, p < 0.05 versus control; *, p < 0.01 versus control. Data are representative of four independent experiments. G, cleaved PARP and cleaved caspase-3 expression levels were detected by Western blotting in peritoneal macrophages from AMPKα1-Lyz-Cre-TG and control mice with or without 7-KC (10 μg/ml), 25-OH cholesterol (10 μg/ml), or staurosporine (0.5 μg/ml) for 16 h. Blots are representative of three independent experiments. H, peritoneal cells from ApoE<sup>−/−</sup> and ApoE<sup>−/−</sup>/AMPKα<sup>−/−</sup> mice were harvested from the peritoneal cavity, and the number of peritoneal cells was counted at the indicated time points after intraperitoneal injections of 1.5 ml of 4% breyer thioglycollate. #, p < 0.01 versus ApoE<sup>−/−</sup> mice at 3 days. *, p < 0.01 versus ApoE<sup>−/−</sup> mice at 4 days (n = 6). I, peritoneal cells from WT and AMPKα1<sup>−/−</sup> mice were harvested by lavaging the peritoneal cavity, and the number of peritoneal cells was counted at the indicated time points after intraperitoneal injections of 1.5 ml of 4% breyer thioglycollate. #, p < 0.05 versus WT mice at 3 days (n = 6). Error bars, S.E.
Figure 3. Deletion of AMPKα1 does not affect the functions of mature macrophages. A, phagocytic capability in bone marrow-derived macrophages from ApoE−/− and ApoE−/−/AMPKα1−/− mice using Invitrogen pHrodo™ E. coli BioParticles® conjugate. Scale bar, 50 μm (n = 3). B, bone marrow-derived macrophages from ApoE−/− or ApoE−/−/AMPKα1−/− mice were placed in the upper wells of the Boyden chamber, with the lower wells containing 100 ng/ml MCP-1. Following incubation at 37 °C for 16 h, migrated cells were counted. Scale bar, 100 μm (n = 5). C, detection of Dil-ox-LDL binding to peritoneal macrophages by fluorescence microscope. Peritoneal macrophages were isolated from ApoE−/− and ApoE−/−/AMPKα1−/− mice at 4 days after thioglycollate injection and incubated with 10 μg/ml Dil-ox-LDL on ice for 1 h. Scale bar, 50 μm (n = 6). D, detection of Dil-ox-LDL binding to mouse bone marrow-derived macrophages by flow cytometry (n = 5). E, peritoneal macrophages were incubated with 50 μg/ml ox-LDL for 48 h, stained with Oil Red O to label intracellular lipid droplets, and photographed under the microscope. Scale bar, 50 μm (n = 5). Error bars, S.E.

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... us into bone marrow after 48 h of M-CSF treatment. The amount of punctate GFP-LC3 structures in ApoE−/−/AMPKα1−/− cells was significantly decreased compared with those in ApoE−/− cells (Fig. 4D). Moreover, inhibition of AMPK with compound C significantly decreased LC3-I/II expression during human monocyte-to-macrophage differentiation (Fig. 4E). Because autophagy is a highly dynamic process, the decreased LC3 expression or the number of GFP-LC3 puncta could be due to either inhibiting autophagy induction or accelerating fusion of autophagosomes with lysosomes. To further explore the role of AMPKα1 in autophagy flux, bone marrow-derived macrophages from WT and AMPKα1−/− mice were treated with or without bafilomycin A1 (5 nM), which blocks the fusion of autophagosomes with lysosomes. As shown in Fig. 4F, bafilomycin A1 increased LC3 accumulation both in WT and AMPKα1−/− macrophages. However, the total LC3 expressions were still lower in AMPKα1−/− macrophages after bafilomycin A1 treatment, suggesting that AMPKα1 may regulate LC3 protein expression from the transcriptional level. We confirmed this result in THP1 cells treated with AMPKα1 siRNA and bafilomycin A1 (Fig. 4G). To determine whether AMPK regulates LC3 at the transcriptional level, mRNA expression of LC3 was examined using real-time RT-PCR in THP1 cells treated with or without AMPKα1 siRNA. As shown in Fig. 4H, the mRNA level of LC3 was significantly decreased in cells transfected with AMPKα1 siRNA. Interestingly, another important autophagy-related kinase, UNC-51-like kinase-1 (ULK1) was also significantly reduced during M-CSF-induced bone marrow differentiation in AMPKα1−/− mice (Fig. 4I). Silence of AMPKα1 by siRNA in THP1 cells decreased both protein level and mRNA level of ULK1 expression (Fig. 4, J and K).

Forkhead box protein O3 (FoxO3) regulates AMPKα1-dependent autophagy induction during monocyte-to-macrophage differentiation

To identify the signaling pathways responsible for regulation of autophagy-related genes by AMPKα1, we focused on the role of FoxO3, which is known to be directly regulated by AMPK (27) and controls the transcription of autophagy-related genes, including LC3 and ULK1 (28, 29). First, we detected the protein expression of FoxO3a in bone marrow-derived macrophages after 5 days of M-CSF treatment or peritoneal macrophages in WT or AMPKα1−/− mice. As shown in Fig. 5, A and B, FoxO3a expression dramatically decreased in AMPKα1−/− macrophages. We further confirmed this finding in THP1 cells transfected with AMPKα1 siRNA (Fig. 5C). To determine whether AMPKα1 regulates FoxO3 at the transcriptional level, mRNA
expression of FoxO3 was detected in THP1 cells transfected with AMPKα1 siRNA. Silence of AMPKα1 significantly decreased FoxO3 mRNA expression (Fig. 5D). On the other hand, overexpression of AMPKα1 in HEK293T cells significantly increased luciferase activity of the FoxO3 promoter (Fig. 5E). To demonstrate that FoxO3 drives LC3 gene expression, mRNA and protein levels of LC3 were detected in THP1 cells transfected with control siRNA or FoxO3a siRNA. Silence of FoxO3a significantly suppressed LC3 mRNA and protein expression (Fig. 5, F and G). In addition, inhibition of FoxO3a by siRNA also suppressed ULK1 mRNA and protein expressions (Fig. 5, H and I).

**Autophagy activation by rapamycin promotes monocyte differentiation in AMPKα1-deleted mice through up-regulation of FoxO3 activity**

Because autophagy is a key process that is regulated by mammalian target of rapamycin (mTOR) signaling and there is interplay between mTORC1, mTORC2, and FoxO proteins (30), we next tested whether rapamycin treatment can increase autophagy induction via up-regulation of FoxO3 activity. Rapamycin treatment significantly increased the transcriptional activity of FoxO3a evaluated by a luciferase assay (Fig. 6A). Moreover, rapamycin also increased LC3 and ULK1 expressions in peritoneal macrophages and bone marrow-derived...
monocytes in WT or AMPKα1−/− mice were isolated 3 days after thioglycollate injection in WT or AMPKα1−/− mice. Protein expression of Foxo3a was detected by Western blotting. #, p < 0.01 versus WT. Data are representative of three independent experiments. E, HEK293T cells were co-transfected with pGL3-enhancer FoxO3a promoter vector in combination with pCMV-AMPKα1 plasmid or control plasmid. Renilla was used as an internal control. After 24 h of transfection, the cells were analyzed for firefly luciferase and Renilla luciferase activity using the Dual-Luciferase reporter assay. Data are expressed as the -fold increase over mock-transfected cells. #, p < 0.01 versus control (n = 6). F, THP1 cells were transfected with FoxO3a siRNA or control siRNA for 48 h, and real-time RT-PCR was performed to quantify mRNA levels of FoxO3a. #, p < 0.01 versus control (n = 6). G, THP1 cells were transfected with FoxO3a siRNA or control siRNA for 48 h, and real-time RT-PCR was performed to quantify mRNA levels of LC3. #, p < 0.01 versus control siRNA (n = 6). H, THP1 cells were transfected with FoxO3a siRNA or control siRNA for 48 h, and ULK1 protein expressions were detected by Western blotting. #, p < 0.01 versus control siRNA. Data are representative of three independent experiments. I, THP1 cells were transfected with FoxO3a or control siRNA for 48 h, and real-time RT-PCR was performed to quantify mRNA levels of ULK1. #, p < 0.01 versus control (n = 6). Error bars, S.E.

Deletion of AMPKα1 suppresses atherosclerotic lesion formation in vivo

To further investigate the effects of AMPKα1 deletion on atherogenesis, age- and gender-matched ApoE−/−/AMPKα1−/− and ApoE−/− mice were fed a Western diet for 10 weeks. As depicted in Fig. 7, A and B, the areas of aortic lesions in both aortic roots and aortic arches were significantly lower in ApoE−/−/AMPKα1−/− mice than those in ApoE−/− mice, indicating that AMPKα1 deletion reduces aortic lesions. Consistently, the necrosis core in the aortic root lesion was significantly smaller in ApoE−/−/AMPKα1−/− than that in ApoE−/− mice (Fig. 7C).

The serum levels of TNF-α, IL-1β, IL-6, MCP-1, and M-CSF were similar between ApoE−/−/AMPKα1−/− and ApoE−/− mice after 5 days of M-CSF treatment. The serum levels of TNF-α, IL-1β, IL-6, MCP-1, and M-CSF were similar between ApoE−/−/AMPKα1−/− and ApoE−/− mice. However, the percentage of Ly6Clow/F4/80high macrophages in ApoE−/−/AMPKα1−/− mice was significantly higher than that in ApoE−/− mice (64.9% versus 32.7%, p < 0.05), suggesting that rapamycin rescues AMPKα1 deletion-induced impairment of monocyte differentiation. Therefore, rapamycin restored the number of Ly6Clow/F4/80high peritoneal macrophages in ApoE−/−/AMPKα1−/− mice (Fig. 6E).
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Figure 6. Autophagy activation by rapamycin promotes monocyte differentiation in AMPKα1−/−-deleted mice through up-regulation of FoxO3 activity.

A, HEK293T cells were transfected with pGL3-enhancer FoxO3a promoter vector for 24 h. The cells were then treated with 500 nM rapamycin for 4 h. Firefly luciferase and Renilla luciferase activity were detected by using the Dual-Luciferase reporter assay. #, p < 0.01 versus control (n = 3). B, peritoneal macrophages were isolated 3 days after thioglycollate injection in WT or AMPKα1−/− mice. The cells were treated with or without 500 nM rapamycin for 24 h. Protein expressions of FoxO3a and LC3 were detected by Western blotting. #, p < 0.05 versus WT with vehicle treatment; *, p < 0.05 versus AMPKα1−/− with vehicle treatment. Data are representative of three independent experiments. C, bone marrow-derived macrophages from WT or AMPKα1−/− mice were treated with vehicle or 250 nM rapamycin for 6 h. #, p < 0.05 versus WT with vehicle treatment; *, p < 0.05 versus AMPKα1−/− with vehicle treatment. Data are representative of three independent experiments. D, detection of Ly6C−/F4/80+ and Ly6C+/F4/80+ cells by flow cytometry in peritoneal cells after 3 days of thioglycollate injection in ApoE−/− and ApoE−/−/AMPKα1−/− mice, which were subcutaneously injected with vehicle or 6 mg/kg/day rapamycin for 10 days; #, p < 0.05 versus Ly6C−/F4/80+ ApoE−/− cells; *, p < 0.01 versus Ly6C−/F4/80+ ApoE−/−/AMPKα1−/− cells; †, p < 0.05 versus F4/80+ ApoE−/− cells; ‡, p < 0.01 versus F4/80+ ApoE−/−/AMPKα1−/− cells (n = 3–5). E, macrophage (F4/80+) numbers in peritoneal cavity 3 days after thioglycollate injection in ApoE−/− and ApoE−/−/AMPKα1−/− mice, which were subcutaneously injected with vehicle or 6 mg/kg/day rapamycin for 10 days. *, p < 0.01 versus vehicle treatment in ApoE−/− mice (n = 3–5). Error bars, S.E.

mice (Fig. 7D). Furthermore, the metabolic parameters, including body weight, blood glucose, total plasma cholesterol, and plasma triglycerides, did not differ between the two groups (Table 1).

Next, we assayed the macrophage contents in aortic root lesions by staining of F4/80, a macrophage-specific marker. As shown in Fig. 7F, the positive area of F4/80 staining is significantly decreased in ApoE−/−/AMPKα1−/− mice when compared with their ApoE−/− counterparts, suggesting that the reduction of macrophages may contribute to smaller aortic lesions in ApoE−/−/AMPKα1−/− mice.

Myeloid AMPKα1 regulates the formation of aortic lesions

To further establish the contributions of AMPKα1 in monocytes/macrophages to the mediation of atherosclerosis, bone marrow from ApoE−/− mice was transferred into ApoE−/−/AMPKα1−/− or ApoE−/− mice versa. The absence of AMPKα1 in bone marrow cells was confirmed by PCR in ApoE−/− mice that received bone marrow from ApoE−/−/AMPKα1−/− or ApoE−/− mice (Fig. 7F). Aortic lesion areas in ApoE−/− mice were significantly reduced after being transplanted with bone marrow from ApoE−/−/AMPKα1−/− mice (Fig. 7G). Conversely, aortic lesions in ApoE−/−/AMPKα1−/− mice were significantly increased after being transplanted with bone marrow from ApoE−/− mice (Fig. 7G). We further generated myeloid-specific AMPKα1-deficient ApoE−/− mice (ApoE−/−/AMPKα1(fl/fl)/LysMCre+). Deficiency of AMPKα1 in myeloid cells did not affect plasma lipid levels (data not shown) but significantly reduced Western diet-induced atherosclerotic lesion areas in the aortic root (Fig. 7H).

Decreased macrophage numbers and increased macrophage apoptosis without change in macrophage proliferation in the aortic lesion of ApoE−/−/AMPKα1−/− mice

We further determined the number of monocytes and macrophages in the aortas of ApoE−/−/AMPKα1−/− and ApoE−/− mice that were fed a Western diet for 10 weeks. There was no change in total Ly6Ch/F4/80+ monocyte numbers between the two groups, but Ly6C+/F4/80+ macrophage numbers in the aortas of ApoE−/−/AMPKα1−/− mice were significantly decreased (Fig. 7I), suggesting that the reduced atherosclerotic lesion size in ApoE−/−/AMPKα1−/− mice might be due to the lesser number of differentiated macrophages in intima. In addition, apoptotic macrophages were significantly increased in the lesions of ApoE−/−/AMPKα1−/− mice (Fig. 7J), as detected by TUNEL and CD68 co-staining. Further, deletion of AMPKα1 showed no effect on macrophage proliferation.
in lesions by analyzing positive immunostaining for proliferation marker Ki67 and macrophage marker Moma2 (Fig. 7K).

**Deletion of AMPKα1 abolished anti-atherosclerotic effects of rapamycin**

A number of studies indicate that the mTOR inhibitor rapamycin has pleiotropic anti-atherosclerotic effects without changes in lipid profile (31–35). Because rapamycin improves AMPKα1 deletion-induced impairment of monocyte-to-macrophage differentiation (64.9% in ApoE−/−/AMPKα1−/− versus 32.7% in ApoE−/−, p < 0.05), we next determined whether rapamycin-promoted monocyte differentiation in ApoE−/−/AMPKα1−/− mice accentuated atherosclerosis. To this end, ApoE−/−/AMPKα1−/− and ApoE−/− mice were fed with a Western diet with or without rapamycin for 10 weeks. Rapamycin had no effect on the serum lipid profile of ApoE−/− or ApoE−/−/AMPKα1−/− mice (data not shown). Consistent with earlier studies (31–35), rapamycin significantly decreased atherosclerotic lesion in aortic roots in ApoE−/− mice (Fig. 7L). However, the suppressing effects of rapamycin on the aortic lesion were ablated in ApoE−/−/AMPKα1−/− mice (Fig. 7L). Taken together, these results suggest that promoting monocyte differentiation by rapamycin in ApoE−/−/AMPKα1−/− mice contributed to increased levels of atherosclerosis in ApoE−/− mice *in vivo*.

**Discussion**

In the present study, we have for the first time demonstrated that AMPKα1 activation promotes monocyte-to-macrophage differentiation and survival by promoting autophagy. We further demonstrate that AMPKα1 controls autophagy by regulating the FoxO3a transcription factor activity, which is involved in the regulation of autophagy-related genes. Consistently, deletion of AMPKα1 reduced plaque growth and intimal macrophage accumulation in the early lesions of ApoE−/− mice. Results from bone marrow transplantation experiments and myeloid specific knock-out of AMPKα1 further suggest that myeloid AMPKα1 plays an important role in atherosclerotic formation. Further, aortic lesions in both aortic roots and aortic arches are significantly lower in ApoE−/−/AMPKα1−/− mice than those in ApoE−/− mice. Importantly, rapamycin treatment, which normalized monocytes differentiation and macrophage survival in ApoE−/−/AMPKα1−/− mice, ablated its atherosclerosis-suppressing effects in ApoE−/− mice. Overall, our results suggest that AMPKα1-dependent and autophagy-mediated monocyte differentiation and survival promote the initiation and progression of atherosclerosis in ApoE−/− mice *in vivo*.

The induction of autophagy is known to be critical for M-CSF-induced monocyte-to-macrophage differentiation (14, 15). There are numerous studies demonstrating the role for AMPK in autophagy induction in response to various cellular stresses (26, 36, 37). M-CSF and its receptor, CD115, are critical for monocyte-to-macrophage differentiation and cell survival (38–40). However, the molecular mechanism underlying how AMPK regulates autophagy in monocyte differentiation has not yet been fully understood. In this study, we show that ox-LDL, 7-KC, and M-CSF increased the conversion of LC3-II, which indicates the induction of autophagy. Consistently, we found that M-CSF, PMA, and ox-LDL, all of which promote the differentiation of monocytes into macrophages, effectively activated AMPK in macrophages. Furthermore, we demonstrate that M-CSF-activated AMPK is via M-CSF receptor-dependent ROS production. Importantly, deletion of AMPKα1 significantly suppressed LC3 and ULK1 mRNA and protein expressions via FoxO3 transcription factor. Autophagy activation with rapamycin improved monocyte differentiation in AMPKα1−/− mice, confirming that deficient autophagy is responsible for deficient differentiation and survival observed in AMPKα1−/− mice. Our findings provide convincing evidence to support a

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**Table 1**

| Parameter          | ApoE−/− | ApoE−/−/AMPKα1−/− |
|--------------------|---------|------------------|
| Body weight (g)    | 29.4 ± 0.9 | 30.3 ± 2.2  |
| Cholesterol (mg/dl)| 903.2 ± 30.2 | 870.7 ± 44.0 |
| Triglyceride (mg/dl)| 139.6 ± 19.4 | 167.1 ± 14.4 |
| Glucose (mg/dl)    | 144.8 ± 5.6  | 161.4 ± 6.2  |
| Systolic BP (mm Hg)| 146 ± 11    | 149 ± 15     |
| Diastolic BP (mm Hg)| 114 ± 8    | 93 ± 8       |

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**Figure 7. AMPKα1 deletion reduces atherosclerotic lesions *in vivo*.** A, representative photomicrographs and quantitative analysis of atherosclerotic lesions in the aortic root of ApoE−/− and ApoE−/−/AMPKα1−/− mice fed a Western diet for 10 weeks. #, p < 0.01 versus ApoE−/− mice; scale bar, 500 μm (n = 15 in the ApoE−/− group and n = 19 in the ApoE−/−/AMPKα1−/− group). B, representative photomicrographs and quantitative analysis of atherosclerotic lesion size in the aortic arch by Sudan IV staining. #, p < 0.01 versus ApoE−/− mice; scale bar, 500 μm (n = 8–9). C, necrotic core region was determined as acellular area of the lesions, lacking nuclei and cytoplasm, from H&E-stained sections. #, p < 0.01 versus ApoE−/− mice. n = 7 in ApoE−/−/AMPKα1−/− mice, and n = 8 in ApoE−/−/AMPKα1−/− mice. D, cytokines in the serum of ApoE−/− and ApoE−/−/AMPKα1−/− mice were measured using the Millipore multiplex kit (n = 8–11). E, representative photomicrographs and quantitative analysis of F4/80-positive areas in the aortic root of ApoE−/− and ApoE−/−/AMPKα1−/− mice. #, p < 0.01 versus ApoE−/− mice. Scale bar, 500 μm, n = 3–4. F, detection of the disruption of AMPKα1 in bone marrow cells by bone marrow transplantation. The genomic DNA of bone marrow cells that were isolated from ApoE−/− mice transplanted with ApoE−/−/AMPKα1−/− or ApoE−/− bone marrow was subjected to PCR analysis, which was performed at 10 weeks after transplantation. G, representative photomicrographs and quantitative analysis of atherosclerotic lesion size in the aortic root by Oil Red O staining in mice transplanted with either ApoE−/− or ApoE−/−/AMPKα1−/− bone marrow. #, p < 0.05 versus ApoE−/− recipient mice that received ApoE−/− bone marrow. #, p < 0.05 versus ApoE−/−/AMPKα1−/− recipient mice that received ApoE−/− bone marrow. Scale bar, 500 μm (n = 9–12). H, representative photomicrographs and quantitative analysis of atherosclerotic lesions in the aortic root of ApoE−/− (AMPKα1−/−) and ApoE−/−/AMPKα1−/− mice fed a Western diet for 24 weeks. #, p < 0.05 versus ApoE−/− mice. Scale bar, 500 μm (n = 6). I, cells digested from whole aortas of ApoE−/− and ApoE−/−/AMPKα1−/− mice fed with 10 weeks of Western diet were stained and sorted by flow cytometry. Monocytes were identified as live, Ly6C+/Ly6C+/Ly6C+/CD11b−, and CD45− cells, and macrophages were identified as live, Ly6C+/Ly6C+/Ly6C+/CD11b+, and CD45− cells. #, p < 0.05 versus ApoE−/− mice (n = 6). J, in situ TUNEL staining of aortic root lesions of Western diet-fed ApoE−/− and ApoE−/−/AMPKα1−/− mice. TUNEL-positive nuclei per 100 CD68-positive cells in the aortic root lesion area were quantified. Magnification, ×400. #, p < 0.01 versus ApoE−/− mice. Scale bar, 50 μm (n = 4). K, representative co-staining images of proliferation marker Ki67 and macrophage marker MOMA-2 in aortic root lesions and quantification of the number of Ki67+ cells per 100 MOMA-2 cells in the lesions. Scale bar, 50 μm (n = 4–5). L, quantitative analysis of atherosclerotic lesions in the aortic root of ApoE−/− or ApoE−/−/AMPKα1−/− mice fed with vehicle or rapamycin for 10 weeks. #, p < 0.05 versus vehicle ApoE−/− mice (n = 6–11). Error bars, S.E.
promoting role of AMPKα1 in controlling autophagy induction in monocyte-to-macrophage differentiation and survival.

A recent study reported that metformin or AICAR inhibits monocyte differentiation into macrophage and therefore attenuates Ang-II-induced atheromatous plaque formation (19). Although the authors showed that metformin or AICAR activated AMPK, it could not exclude the AMPK-independent effects of metformin or AICAR on monocyte differentiation. In fact, we found that metformin protected against hyperglycemia-induced atherosclerosis through AMPKα2 activation, which has no effect on monocyte differentiation (41). In contrast with a reduced atherosclerosis observed in this study, Cao et al. (42) reported that myeloid AMPKα1-deleted LDL receptor knock-out mice (Ldlr−/−) increased formation of atherosclerotic plaque with enhanced macrophage inflammation and higher plasma triglyceride and cholesterol content. Although ApoE−/− and Ldlr−/− mice have been used extensively as two mouse models of atherogenesis, Ldlr−/− mice have much lower plasma cholesterol levels and develop less severe atherosclerotic lesions than ApoE−/− mice on a normal chow diet (43, 44). Therefore, the elevation of lipid level by deletion of myeloid AMPKα1 in Ldlr−/− mice may have a major contributor to the manifestation of atherosclerosis in this mouse model. Indeed, pharmacological AMPK activator S17834 has been reported to attenuate hyperlipidemia and suppress aortic atherosclerosis in insulin-resistant Ldlr−/− mice in part through suppression of SREBP-1c- and SREBP-2-dependent lipogenesis (45).

In our models, AMPKα1 deletion or myeloid AMPKα1 deletion in ApoE−/− background did not alter lipid levels. Therefore, the impaired monocyte differentiation and increased monocyte/macrophage apoptosis due to AMPKα1 deletion may play the key roles in alleviation of atherosclerosis in an ApoE knock-out mouse model. Overall, our results indicate that AMPKα1 activation during monocyte differentiation via its promotion of monocyte differentiation and survival increases the numbers of macrophage, which promotes the initiation and progression of atherosclerosis in ApoE−/− mice. Why AMPKα1 deletion has opposite impacts in plasma lipid levels in ApoE−/− mice versus LDLr−/− mice is unknown and warrants further investigation.

A growing body of evidence indicates that the mTOR inhibitor rapamycin has pleiotropic anti-atherosclerotic effects. Administration of rapamycin systemically has been demonstrated to prevent the development of atherosclerosis in different animal models (31–35). Although the exact mechanisms accounting for the beneficial effects of mTOR inhibition on atherosclerosis are not yet clear, the potential beneficial effects of rapamycin and its derivatives include inhibition of smooth muscle cell proliferation (46), reduced MCP-1 expression within the injured arterial wall (34), and inhibition of lipid accumulation in macrophages and smooth muscle cells (47, 48). Our current results also show that although rapamycin slightly promoted monocyte differentiation, the net effect of rapamycin treatment was anti-atherosclerotic in ApoE−/− mice, suggesting that multiple mechanisms may be involved in the regulation of atherosclerosis development. However, in ApoE−/−/AMPKα1−/− mice, the percentage increase in monocyte differentiation into macrophage was significantly higher than that of ApoE−/− mice when compared with vehicle treatment, and rapamycin treatment showed no effect on plaque size in ApoE−/−/AMPKα1−/− mice, implying that improved differentiation by rapamycin in ApoE−/−/AMPKα1−/− mice may counteract the other anti-atherosclerosis effects of rapamycin.

In summary, we demonstrate that AMPKα1 plays a key role in modulating monocyte-to-macrophage differentiation and survival through FoxO3-dependent autophagy induction by regulating expression of genes involved in autophagy. AMPKα1 activation accelerates monocyte differentiation and survival, which participates in the initiation and progression of atherosclerosis.

**Experimental procedures**

**Animals**

The animal protocol and procedures were reviewed and approved by the University of Oklahoma Institute Animal Care and Use Committee.

**Generation of ApoE and AMPKα1 double-knock-out mice**

AMPKα1−/− mice that had been generated and backcrossed onto a C57BL/6 background, as described previously (49), were crossed with ApoE−/− mice (Jackson Laboratory) to generate ApoE−/−/AMPKα1−/− mice. Mice were housed in temperature-controlled cages under a 12-h light-dark cycle and given free access to water and chow. Accelerated atherosclerosis was induced by feeding the mice with a Western diet containing 0.21% cholesterol and 21% fat (Research Diets Inc., D12079B). This diet was administered at 8 weeks of male mice and continued for 10 weeks. In some experiments, mice were fed with either a microencapsulated rapamycin-containing Western diet, which contains 14 mg/kg food of rapamycin providing a dose of ~2.24 mg of rapamycin/kg of body weight/day (50, 51), or a control Western diet (with empty microcapsules) for 10 weeks. Microencapsulated rapamycin and empty microcapsules were purchased from Rapamycin Holdings Inc. (San Antonio, TX) and incorporated into the Western diet by Research Diets Inc.

**Generation of ApoE and macrophage-specific AMPKα1 double-knock-out mice**

The floxed AMPKα1 mice (AMPKα1fl/fl, stock number 014141) and LysMCre+ mice (stock number 004781) were obtained from the Jackson Laboratory. ApoE−/−/AMPKα1fl/fl/LysMCre+ mice were obtained by crossing of AMPKα1fl/fl mice with LysMCre+ mice and then back-crossing with ApoE−/− mice. ApoE−/−/AMPKα1fl/fl/LysMCre+ mice were used as control mice. The Western diet was administered to male mice at 8 weeks and continued for 24 weeks.

**Generation of myeloid-specific AMPKα1 transgenic mice**

The AMPKα1 cDNA (Addgene, catalog no. 27297) was subcloned into the CAG-CAT-LacZ construct containing a cytomegalovirus enhancer and a chicken β-actin gene promoter, linked to the chloramphenicol acetyltransferase (CAT) gene, and flanked by loxp sites, which were linearized, and then microinjected into fertilized mouse embryos to produce...
AMPKα1 flox transgenic mice. The AMPKα1 flox transgenic mice were identified by PCR analysis of tail genomic DNA using the following primers: forward, 5′-CCCCCTGAACTGTGAAA-CATAAAATG-3′; reverse, 5′-CGGCTATCTTTGCTTCAATTGTGTG-3′. In these mice, AMPKα1 expression is blocked by the presence of the CAT gene; when the CAT sequence is excised by Cre recombinase, AMPKα1 is expressed. AMPKα1 flox transgenic mice were crossed with mice carrying LysM-Cre to yield conditional AMPKα1-Cre-TG mice, which were identified by using the following primers: forward, 5′-GCTATCTTTGCTTGCAATGTGTG-3′; reverse, 5′-CGGCTATCTTTGCATCTGATGTG-3′.

**Monocyte AMPKα1 in atherosclerosis**

AMPKα1-exudate was isolated. In some experiments, 4% thioglycollate. After 1–4 days of injection, the peritoneal cells were collected on day 10. Peritoneal monocytes were differentiated into mature macrophages after 3 days (15, 20). To investigate the effect of AMPKα1 on monocyte-to-macrophage differentiation, mice were intraperitoneally injected with 1.5 ml of 4% thioglycollate. After 1–4 days of injection, the peritoneal monocytes were subcutaneously injected with rapamycin or vehicle for 10 days (6 mg/kg/day). On day 7, the mice were injected with 1.5 ml of 4% thioglycollate, and the peritoneal cells were collected on day 10. Peritoneal monocytes were identified as Ly6C+b220loF4/80hi in CD11bhiCD90loB220loCD49blo cells. Mice were retro-orbitally injected with 1-Cre-TG mice, which were identified by PCR analysis of tail genomic DNA using the following primers: forward, 5′-CCCCCTGAACCTGAAA-1-Cre-3′, reverse, 5′-ATCGTGCGAGAGGGCG-3′/H11032-Cre-3′.

**Monocyte-to-macrophage differentiation in vivo**

Thioglycollate-recruited monocytes in the peritoneum are differentiated into mature macrophages after 3 days (15, 20). To investigate the effect of AMPKα1 on monocyte-to-macrophage differentiation, mice were intraperitoneally injected with 1.5 ml of 4% thioglycollate. After 1–4 days of injection, the peritoneal exudate was isolated. In some experiments, ApoE−/−/AMPKα1−/− and ApoE−/− mice were subcutaneously injected with rapamycin or vehicle for 10 days (6 mg/kg/day). On day 7, the mice were injected with 1.5 ml of 4% thioglycollate, and the peritoneal cells were collected on day 10. Peritoneal monocytes were identified as Ly6C+b220loF4/80hi in CD11bhiCD90loB220loCD49bloNK1.1loLy-6Glo cells, and macrophages were identified as Ly6C+b220loCD11bhiCD90loB220loCD49bloNK1.1loLy-6Glo cells by flow cytometry.

**Monocyte isolation and monocyte-to-macrophage differentiation in vitro**

Human peripheral blood mononuclear cells were separated by Ficoll density gradient centrifugation from venous blood of healthy consenting volunteers, after approval from the ethical committee of the University of Oklahoma Health Sciences Center. Lymphocyte-monocyte-rich layer was washed with PBS and then suspended in DMEM supplemented with 2 mM l-glutamine, 100 units/ml penicillin/streptomycin, and 10% FBS for 2 h at 37 °C and. Non-adherent cells were then removed, and the adherent cells were cultured in DMEM supplemented with 2 mM l-glutamine, 100 units/ml penicillin/streptomycin, 10% FBS serum, and 40 ng/ml human M-CSF in the presence or absence of AMPK inhibitor compound C for the indicated time.

Blood from 10–12 weeks old ApoE−/−/AMPKα1−/− and ApoE−/− mice was collected with heparin, and red blood cells were removed by ammonium chloride lysis. The monocytes were then enriched using the immunomagnetic EasySep® mouse monocyte enrichment kit (STEMCELL Technologies Inc.) according to the manufacturer’s instructions. The purity of CD11b+Ly6C+ cells was assessed by flow cytometry and ranged from 75 to 85%.

THP-1 cells were cultured in RPMI 1640 with 10% FBS. The cells were differentiated with PMA, ox-LDL, or PMA and ox-LDL combinations with or without compound C for 3 days.

**Bone marrow transplantation**

Eight-week-old male ApoE−/− and ApoE−/−/AMPKα1−/− mice were subjected to 11-gray lethal total-body irradiation (two doses of 5.5 grays within an interval of 4 h) to eliminate endogenous bone marrow stem cells and bone marrow-derived cells. Mice were retro-orbitally injected with ApoE−/− or ApoE−/−/AMPKα1−/− bone marrow cells (5 × 106 cells). Four weeks after transplantation, the mice were placed on the Western diet for 8 weeks. The hematologic chimerism of transplanted ApoE−/− mice was confirmed in genomic DNA from bone marrow by PCR analysis.

**Atherosclerotic lesion analysis**

After being fed the Western diet for 10 weeks, the mice were fasted for 14 h and then anesthetized and euthanized. The heart and aortic tissues were removed from the ascending aorta to the ileal bifurcation and placed in 4% paraformaldehyde for 24–48 h. After fixation, the adventitia was thoroughly cleaned under a dissecting microscope. To analyze the lesion area in the aortic root, the heart was dissected from the aorta, embedded in OCT compound, and sectioned (8-μm thickness). Four serial cryosections were collected from each mouse and stained with Oil Red O for neutral lipids and then counterstained with hematoxylin to visualize nuclei. Images of plaques were captured under the Olympus microscope, which was connected to a QImaging Retiga CCD camera, and quantitative analysis was performed with Image J software (National Institute of Health) by averaging the lesion areas in the four sections. To analyze the lesion area in the aortic arch, the intimal surface was exposed by a longitudinal cut from the ascending arch to an area that was 5 mm distal to the left subclavian artery. This allowed the lumen of the aortic arch to be laid flat. The aorta was rinsed for 5 min in 75% ethanol, stained with 0.5% Sudan IV in 35% ethanol and 50% acetone for 15 min, destained in 75% ethanol for 5 min, and then rinsed with PBS. Digital images of the aorta were captured under a stereomicroscope, and the lesion area was quantified from the aortic arch to the area that was 5 mm distal to the left subclavian.

**Macrophage staining and macrophage proliferation in situ**

Staining of macrophages in aortic root lesion was performed with rat anti-mouse F4/80 from Abcam. Apoptotic macrophages in atherosclerotic lesions were detected by using the DeadEnd™ fluorometric TUNEL system (Promega) and rat anti-mouse CD68 primary antibody (AbD Serotec) followed by goat anti-rat IgG-conjugated Alexa Fluor 594.

To detect in situ macrophage proliferation, sections of aortic root were labeled with a primary rat anti-mouse MOMA-2 antibody (AbD Serotec) and a rabbit anti-mouse antibody, Ki-67 (Abcam), detected by Alexa Fluor 488 goat anti-rabbit IgG antibody and Alexa Fluor 594 donkey anti-rabbit IgG antibody (Life Technologies, Inc.), respectively. Images were recorded using a Zeiss LSM 710 inverted confocal microscope.

**Determination of serum cholesterol, triglyceride, and blood glucose levels**

Blood glucose levels were determined by applying tail blood to a OneTouch Ultra blood glucose monitoring system (LifeScan). Serum cholesterol and triglyceride levels were measured enzymatically using Infinity reagents from Thermo Scientific, according to the manufacturer’s instructions.
Mouse peritoneal macrophages and bone marrow-derived macrophages

ApoE⁻/⁻/AMPKα1⁻/⁻ or ApoE⁻/⁻ mice (8–10 weeks old) were intraperitoneally injected with 1.5 ml of 4% BBL thiglycollate brewer (BD Biosciences). After 4 days, peritoneal macrophages were collected from peritoneal exudates. Cells were cultured in DMEM (Corning Cellgro), which was supplemented with 2 mM l-glutamine, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 10% FBS for 2 h. Non-adherent cells were removed by washing with PBS, and adherent cells were taken as peritoneal macrophages. To obtain bone marrow-derived macrophages, mouse femurs and tibias were isolated, and both ends of the bones were cut with scissors. The cells from the bone marrow were flushed with 10 ml of DMEM. Cells were seeded at a density of 2 × 10⁵ marrow cells/cm² in 6-well culture plates in DMEM containing 10% FBS, penicillin, streptomycin, 2 mM l-glutamine, and 40 ng/ml mouse M-CSF (eBioscience). After 3 days, the medium was replaced with fresh medium. After another 6 days in culture, differentiated macrophages were used for various experiments in DMEM with 10% FBS.

Phagocytosis assay

The phagocytic activity of macrophages was measured by the uptake of red fluorescent pHrodo E. coli bioparticles (Invitrogen). Briefly, 5 × 10⁴ macrophages were suspended in 100 µl of Hanks’ balanced salt solution containing 20 mM HEPES, pH 7.4, and mixed with 20 µl of pHrodo E. coli bioparticles. The mixture was incubated for 30 min at either 37 °C for uptake activity or 0 °C for background activity.

Chemotaxis assay

Macrophage chemotaxis assays were performed using 8-µm Corning transwells. Bone marrow-derived macrophages from ApoE⁻/⁻/AMPKα1⁻/⁻ or ApoE⁻/⁻ mice were placed in the upper wells of the chamber, and the lower wells contained 100 ng/ml MCP-1. Following incubation at 37 °C for 16 h, cells remaining on the upper surface of the filter were removed mechanically. Migrated cells were stained with hematoxylin and then counted manually under the microscope.

Binding of ox-LDL to macrophages

Peritoneal macrophages or bone marrow-derived macrophages were plated on 12-well plates at a concentration of 2.5 × 10⁵ cells/well. The cells were incubated with 10 µg/ml DiL-oxLDL (Kalen Biomedical) on ice for 1 h and washed three times with ice-cold PBS. DiL-oxLDL binding was assayed by either fluorescence microscopy or flow cytometry using excitation and emission wavelengths of 514 and 565 nm, respectively.

Foam cell formation assay

Peritoneal macrophages from ApoE⁻/⁻/AMPKα1⁻/⁻ or ApoE⁻/⁻ mice were incubated with either 50 µg/ml acetylated-LDL or 50 µg/ml ox-LDL for 48 h. After incubation, the cells were washed, fixed with 4% paraformaldehyde, stained with Oil Red O to detect intracellular neutral lipids, counterstained with hematoxylin, and photographed under the microscope.

Flow cytometry assay

ApoE⁻/⁻/AMPKα1⁻/⁻ and ApoE⁻/⁻ mice, which were fed a Western diet for 10 weeks, were anesthetized and perfused by cardiac puncture with Dulbecco’s PBS containing 2 mM EDTA. Aortas were cut from the heart to the abdominal bifurcation, cleaned, and digested with 125 units/ml collagenase type XI, 60 units/ml hyaluronidase type I-s, 60 units/ml DNase I, and 450 units/ml collagenase type I in PBS containing 20 mM HEPES at 37 °C for 1 h. The digested aortas were gently forced through a 70-µm strainer to obtain a single-cell suspension. Cells were incubated with FITC-CD14, phcoerythrin lineage antigen, allophycocyanin-F4/80, and peridinin-chlorophyll-protein complex-cy5.5-CD11b antibodies for 20 min at 4 °C and analyzed by flow cytometry on a FACSCalibur system. Macrophages were identified as live, F4/80lo/CD11b-positive, and monocytes were identified as live, F4/80hi/CD11b⁺, and CD45⁺ cells.

Aortic flow cytometry

Serum levels of TNF-α, IL-1β, IL-6, MCP-1, and M-CSF in ApoE⁻/⁻/AMPKα1⁻/⁻ and ApoE⁻/⁻ mice, which were fed a Western diet for 10 weeks, were measured using the Millipore multiplex kit by the Bio-Plex 200 system (Bio-Rad). MCP-1 levels in the peritoneal lavage and serum after 4 h of thioglycollate injection were measured by the enzyme-linked immunosorbent assay kit (BioLegend), following the manufacturer’s protocol.

Western-blotting analysis

Cell lysates or tissue homogenates were subjected to Western-blotting analysis, as described previously (52).

AMPK activity assay

THP-1 cells were treated with 40 ng/ml M-CSF for the indicated times, and endogenous AMPK was immunoprecipitated using the AMPK antibody. AMPK activity was assayed using the SAMS peptide, as described previously (53).
**Luciferase assay**

HEK293T cells were co-transfected with pGL3-enhancer FoxO3a promoter vector (FHRE-Luc) and Renilla luciferase vector was used as an internal control. After 24 h of transfection, the cells were harvested and analyzed for firefly luciferase and Renilla luciferase activity using the Dual-Luciferase reporter assay (Promega) as per the manufacturer’s protocol. Data were expressed as the -fold increase over mock-transfected cells. HEK293T cells were transfected with pGL3-enhancer FoxO3a promoter vector and Renilla luciferase vector for 24 h. The cells were then treated with or without 500 nM rapamycin for 4 h. Foxp3 promoter activity was measured as described above.

**Real-time RT-PCR**

Total RNA was extracted using the Qiagen RNeasy minikit. The samples were reverse-transcribed using the iScript cDNA synthesis kit (Bio-Rad). The primers used in PCR are as follows: 18S rRNA, forward (5'-TAAAGGATGAGTTCA-3') and reverse (5'-GGCTTGCCTGC-3'); ULK1, forward (5'-GGATGGAGTTCTTC-3') and reverse (5'-CTGTCAATCCTGTC-3'); LC3, forward (5'-TCTCAAGCGGCTTTCAAGC-3') and reverse (5'-CGATGATCACCCGGATTTTG-3'); FoxO3a, forward (5'-CTACGAGTGGATGTCGTT-3') and reverse (5'-TGTGCGGATGGAGTTC-3').

Real-time quantification was performed by SYBR Green (Bio-Rad) with the C1000 thermal cycler, CFX96 detection system (Bio-Rad). Relative gene expression was normalized to 18S rRNA and compared using the ΔΔCt method.

**Statistical analysis**

Data are reported as mean ± S.E. Statistical comparisons were performed with the unpaired Student’s t test to compare two groups or one-way analysis of variance to compare three or more groups. The Bonferroni multiple-comparison test was used for post hoc analysis. p < 0.05 was considered significant.

**Author contributions**—M. Z., H. Z., and Y. D. designed and performed the experiments and analyzed the data with the help of all authors, wrote the manuscript, and acquired partial funding. Z. L., M. Z., H. Z., and Y. D. designed and performed the experiments and analyzed the data with the help of all authors, wrote the manuscript, and acquired partial funding. Z. L., M. Z., H. Z., and Y. D. designed and performed the experiments and analyzed the data with the help of all authors, wrote the manuscript, and acquired partial funding. Z. L., M. Z., H. Z., and Y. D. designed and performed the experiments and analyzed the data with the help of all authors, wrote the manuscript, and acquired partial funding.

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