Apolipoprotein E Enhances MicroRNA-146a in Monocytes and Macrophages to Suppress Nuclear Factor-κB–Driven Inflammation and Atherosclerosis

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Rationale: Apolipoprotein E (apoE) exerts anti-inflammatory properties that protect against atherosclerosis and other inflammatory diseases. However, mechanisms by which apoE suppresses the cellular activation of leukocytes commonly associated with atherosclerosis remain incompletely understood.

Objective: To test the hypothesis that apoE suppresses inflammation and atherosclerosis by regulating cellular microRNA levels in these leukocytes.

Methods and Results: An assessment of apoE expression among such leukocyte subsets in wild-type mice revealed that only macrophages and monocytes express apoE abundantly. An absence of apoE expression in macrophages and monocytes resulted in enhanced nuclear factor-κB signaling and an exaggerated inflammatory response on stimulation with lipopolysaccharide. This correlated with reduced levels of microRNA-146a, a critical negative regulator of nuclear factor-κB signaling. Ectopic apoE expression in Apoe−/− macrophages and monocytes raised miR-146a levels, whereas its silencing in wild-type cells had an opposite effect. Mechanistically, apoE increased the expression of transcription factor purine-rich PU-box–binding protein 1, which raised levels of pri-miR-146 transcripts, demonstrating that apoE exerts transcriptional control over miR-146a. In vivo, even a small amount of apoE expression in macrophages and monocytes of hypomorphic apoE mice led to increased miR-146a levels, and inhibited macrophage proinflammatory responses, Ly-6Chigh monocytosis, and atherosclerosis in the settings of hyperlipidemia. Accordingly, cellular enrichment of miR-146a through the systemic delivery of miR-146a mimetics in Apoe−/−Ldlr−/− and Ldlr−/− mice attenuated monocyte/macrophage activation and atherosclerosis in the absence of plasma lipid reduction.

Conclusions: Our data demonstrate that cellular apoE expression suppresses nuclear factor-κB-mediated inflammation and atherosclerosis by enhancing miR-146a levels in monocytes and macrophages. (Circ Res. 2015;117:e1-e11. DOI: 10.1161/CIRCRESAHA.117.305844.)

Key Words: atherosclerosis • apolipoproteins E • macrophages • microRNAs

A polipoprotein E (apoE), a 34-kDa secreted protein, is well-recognized for its ability to suppress atherosclerosis.1 Beyond its pivotal role in lipoprotein cholesterol transport and in regulating cellular lipid levels, apoE has long been known to exert anti-inflammatory properties. ApoE-deficient (Apoe−/−) mice display enhanced chronic inflammation in response to spontaneous and diet-induced hypercholesterolemia, as well as an exaggerated acute immune response when challenged with bacterial lipopolysaccharide (LPS), which predisposes them to death from sepsis.2,3 Macrophages are an important source of inflammatory cytokine production in the settings of both acute and chronic inflammation, and apoE is known to be highly expressed by macrophages.1 Although the addition of exogenous apoE and its mimetic peptides has been reported to suppress macrophage inflammatory responses4 and induce anti-inflammatory macrophage phenotypes,5 far less is known about how endogenous apoE expression mediates anti-inflammatory functions in macrophages and in other leukocytes.

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Macrophages and circulating monocytes are important effector cells in many inflammatory diseases, including atherosclerosis.6 Induction of inflammatory mediators in these cells is primarily mediated by the activation of the transcription factor nuclear factor-κB (NF-κB) pathway. Several lines of evidence suggest that NF-κB is activated in peripheral blood monocytes and lesional macrophages in patients with acute coronary syndrome and unstable angina.7 Experimental data using animal models have suggested that aberrant NF-κB activation in myeloid cells can contribute to the initiation and progression of atherosclerosis.8,9 Thus, understanding how NF-κB
activity in monocytes and macrophages is controlled in the context of hyperlipidemia is of great interest and may provide new therapeutic avenues for the treatment of atherosclerosis.

Multiple regulatory mechanisms have evolved to limit NF-kB activation in macrophages and monocytes. Of these, microRNA-146a (miR-146a) has emerged as a key negative regulator of NF-kB. In this study, we tested the hypothesis that apoeE expression can suppress NF-kB-mediated inflammation by regulating cellular microRNA levels in macrophages and monocytes. Our findings reveal that cellular apoeE expression affects the levels of several microRNAs, including miR-146a, and thereby provides new insight to understand how apoeE suppresses inflammation and atherosclerosis beyond reducing plasma lipid levels.

Methods

In Vivo Administration of MicroRNA Mimetics
The in vivo delivery of microRNA mimetics was performed as previously described. The uptake of miR-146a mimetics (146a-m) by target cells in vivo was validated using miR-146a–/– mice obtained from the Jackson Laboratories. Briefly, miR-146a–/– mice were injected intravenously via the tail vein with a mixture of Lipofectamine 2000 and mimetics (miRNA negative control, or miR-146a; 1 nmol/mouse) suspended in 200 μL phosphate buffered saline twice a week for 2 weeks. Ly-6Clow monocytes sorted from spleens and macrophages purified from peritoneal lavage were analyzed for miR-146a expression. To study the effects of microRNA mimetics on atherosclerosis, Apoe–/– Ldlr–/– mice (11±1-week-old) were continuously fed a normal chow diet and injected with microRNA mimetics as above for a period of 6 weeks. To control for endogenous apoeE expression on miR-146a effects in atherosclerosis, the study was repeated with 10-week-old Ldlr–/– mice fed a high-fat diet. After 2 weeks of diet initiation, the mice were treated with 146a-m or controls as above for a period of 6 weeks. Ly-6Chigh monocytes sorted from spleens and macrophages,11 its expression pattern among other leukocyte subsets known to participate in atherosclerosis is not well defined. We explored this question by measuring apoeE mRNA levels in various subsets of mature myeloid and lymphoid cells isolated from wild-type (WT) C57BL/6 mice by quantitative real-time PCR. The mRNA levels of apoeE in these cells were then compared with those in resident peritoneal macrophage set arbitrarily to 100%. As shown in Figure 1A, apoeE expression was found to be restricted to myeloid cells, primarily among monocytes. Interestingly, Ly-6Clow monocytes expressed robust amounts (32.0±5.8) that were on average 5-fold more apoeE mRNA than Ly-6Chigh monocytes (6.7±1.4, P<0.001). Consistent with mRNA expression data, quantification of cellular apoeE protein content by flow cytometry showed that Ly-6Clow monocytes had 2-fold more apoeE than Ly-6Chigh monocytes (Figure 1B). In contrast, apoeE was expressed far less in neutrophils (1.2±0.2), dendritic cell subsets (<1.0), plasmacytoid dendritic cells (<0.5), and B cells (<1.0), and it was undetectable in T lymphocytes (Figure 1A). The strikingly different apoeE expression levels observed between the 2 major monocyte subsets suggest that it may play a role in regulating their functional heterogeneity.

ApoE Expression Reduces NF-kB Inflammatory Signaling in Macrophages and Monocytes
Numerous studies have shown that NF-kB signaling driven by toll-like receptors (TLRs) in monocytes and macrophages is an important contributor to acute and chronic inflammation, including atherosclerosis. However, little is known about the impact that cellular apoeE expression can exert on NF-kB signaling in response to TLR signaling. We addressed this question with cultured macrophages and monocytes exposed to LPS, a well-established TLR4 ligand and activator of NF-kB signaling in these cells. Peritoneal macrophages from 4-week-old WT and Apoe–/– mice were stimulated with LPS and subsequently analyzed for the subcellular distribution and phosphorylation of the major NF-kB subunit p65 by immunofluorescence and flow cytometry. At a basal unstimulated state, resident peritoneal macrophages cultured from either WT or Apoe–/– mice displayed a similarly low level of nuclear p65 (Figure 1D). In contrast, TLR4 activation by LPS stimulation caused a pronounced nuclear translocation of p65 in both WT and Apoe–/– macrophages (Figure 1C and 1D). However, macrophages derived from Apoe–/– mice displayed almost 20% more nuclei containing p65 than those of WT mice (Figure 1C and 1D). Moreover, we also observed that the absolute cellular levels of p65 phosphorylation were significantly greater among macrophages derived from Apoe–/– mice than those of WT mice (Figure 1E and 1F). We next explored the consequence of increased p65 nuclear localization and phosphorylation on the cellular expression of NF-kB target genes in Apoe–/– macrophages. As shown in Figure 1G and 1H, Apoe–/– macrophages consistently displayed higher mRNA expression levels of tumor necrosis factor α (TNF-α) and interleukin-6 compared with WT macrophages. A similar increase in LPS-induced p65 phosphorylation (Figure 1I) and TNF-α mRNA expression (Figure 1J) was also observed among Ly-6Clo monocytes isolated from Apoe–/– mice compared with those isolated from WT mice. Collectively, our findings show that apoeE expression in macrophages and monocytes restrains NF-kB signaling and downstream inflammatory cytokine expression in response to LPS stimulation.
ApoE Deficiency Results in Reduced miR-146a Expression in Macrophages and Monocytes

We sought to identify the underlying mechanisms by which apoE controls NF-κB activity in macrophages. Several microRNAs are known to differentially regulate NF-κB signaling in macrophages, including miR-146a, miR-147, miR-21, miR-132, miR-125b, and miR-155. We explored whether a loss of apoE expression in macrophages could affect levels of these microRNAs to alter their cellular responsiveness to LPS-induced NF-κB activation. Our results show...
that compared with WT macrophages, Apoe–/– macrophages displayed lower levels of miR-146a, miR-147, and miR-21. However, miR-132, miR-125b, and miR-155 were present at similar levels in these 2 types of macrophages (Figure 2A).

In beginning to explore the relationship between cellular apoE expression and microRNA levels, we focused on miR-146a because of its well established role in suppressing NF-κB–driven inflammatory signaling and cytokine expression in macrophages.10,15 Bone marrow–derived macrophages (BMDM) prepared from 3-week-old Apoe–/– and WT mice were exposed to LPS, and levels of miR-146a were determined by quantitative real-time PCR at various time points after stimulation. A loss of apoE expression in Apoe–/– BMDM led to a 2-fold decrease in miR-146a expression even before LPS stimulation, indicating the importance of apoE expression in the maintenance of baseline miR-146a levels in macrophages (Figure 2B). Although miR-146a levels increased in Apoe–/– BMDM after LPS stimulation, this occurred at a much lower magnitude than in WT BMDM (Figure 2B). Interestingly, the difference in miR-146a levels were even more pronounced at the 6 and 18 hours time points, demonstrating that a loss of apoE impairs the cell’s ability to produce and maintain a normal pool of miR-146a in response to an inflammatory stimulus (Figure 2B). We explored the functional consequences of miR-146a paucity in Apoe–/– macrophages on the expression levels of its target genes, including TNF receptor–associated factor 6 (TRAF6) and interleukin-1 receptor–associated kinase 1 (IRAK1).10,15 Not surprisingly, we noted that the loss of apoE expression in macrophages consistently led to a 2-fold increase in the mRNA levels of these 2 key molecular activators of NF-κB signaling (Figure 2C). These findings therefore explain in part our observations of increased NF-κB activation and proinflammatory cytokine expression in Apoe–/– macrophages (Figure 1).

To test the possibility that hyperlipidemia contributed to the observed reduced miR-146a levels in cells derived from Apoe–/– mice, we repeated our experiments with resident peritoneal macrophages isolated from Ldlr–/– mice that displayed a similar level of hyperlipidemia (Online Figure IA). As shown in Online Figure IB–ID, macrophages isolated from Ldlr–/– mice exhibited similar levels of miR-146a (P=0.18), as well as mRNA levels of TRAF6 (P=0.71) and IRAK1 (P=0.49) as those isolated from WT mice. These data demonstrate that a loss of apoE expression but not the presence of hyperlipidemia is responsible for reduced miR-146a expression and activity in macrophages derived from Apoe–/– mice.

Finally, we tested if other leukocytes derived from Apoe–/– mice were also susceptible to reduced miR-146a levels. Interestingly, we detected significantly reduced amounts of miR-146a in both Ly-6Chigh and Ly-6Clow monocytes derived from Apoe–/– mice compared with those of WT mice at 3 to 4 weeks of age (Figure 2D). However, this effect did not extend to other
ApoE–/– miR-146a expression in (Figure 3B). Remarkably, restoring only 14% of WT apoE mRNA expression levels in Apoe−/− BMDM transduced with a low amount of apoE expression plasmid delivered at 5 ng/mL (Figure 3A) led to a pronounced 40% increase in miR-146a levels (Figure 3B). Interestingly, expressing 14-fold more apoE mRNA expression in cells transduced with 125 ng/mL apoE expression plasmid (Figure 3A) resulted in only a 50% increase in miR-146a levels (Figure 3B). These results suggest that only a small amount of cell-derived apoE expression is required to positively drive an accumulation of miR-146a in macrophages. The induction of miR-146a by apoE expression was even more effective in Ly-6Chigh monocytes which displayed a 3-fold increase in miR-146a levels when transduced with the apoE expression plasmid at 125 ng/mL (Figure 3C).

We also tested whether suppressing apoE expression could have an opposite effect on miR-146a levels in these cells. As shown in Figure 3D, transducing WT mouse monocyte-derived macrophages with an apoE small interfering RNA led to a 40% reduction of miR-146a levels 48 hours after transfection. Together, our in vitro data demonstrate a causative role for cellular apoE expression in the enhancement of miR-146a levels in macrophages and monocytes and that low amounts of cellular apoE expression are sufficient to drive a meaningful biological effect.

**ApoE Increases miR-146a by Enhancing the Transcription Factor PU.1**

We next explored the mechanisms by which cellular apoE expression increases miR-146a levels in macrophages. At first, levels of miR-146a primary transcript (pri-miR146a) were detected to examine whether apoE affects miR-146a transcription. As shown in Figure 4A, peritoneal macrophages from WT mice expressed 2-fold more pri-miR146a than those from Apoe−/− mice. Moreover, transducing apoE expression in Apoe−/− BMDM raised cellular pri-miR-146a levels (Figure 4B). These results suggest that apoE increases miR-146a in macrophages by enhancing its transcription.

miR-146a expression in hematopoietic cells is known to be transcriptionally induced by NF-κB and purine-rich PU-box–binding protein 1 (PU.1).10,16 Because NF-κB activity is similarly low in freshly isolated resident peritoneal macrophages derived from young, 3 to 4-week-old Apoe−/− and WT mice (Figure 1D and 1F), we wondered whether apoE enhances miR-146a transcription by regulating basal PU.1 levels. Indeed, PU.1 mRNA and protein levels were significantly greater in WT macrophages than in Apoe−/− macrophages (Figure 4C and 4D). Furthermore, transduced expression of apoE in Apoe−/− macrophages increased PU.1 mRNA and protein expression (Figure 4E and 4F).

We then investigated the functional role of PU.1 in apoE-induced miR-146a transcription by downregulating its expression. As shown in Figure 4G, the knockdown of PU.1 in WT BMDM by small interfering RNA led to a 50% reduction in pri-miR-146a expression levels. Importantly, the induction of pri-miR-146a by ectopic apoE expression in Apoe−/− BMDM was significantly reduced but not completely abrogated by PU.1 small interfering RNA (Figure 4H). Together, these data demonstrate that apoE drives the transcription of miR-146a in part by enhancing cellular expression of PU.1.
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apoE (by increasing miR-146a in the settings of hyperlipidemia. and macrophage inflammation, and thereby atherosclerosis, examined whether apoE expression could suppress monocyte

Atherosclerosis caused by chronic hyperlipidemia. Both macrophages (Figure 5A) and Ly-6C<sup>high</sup> monocytes (Figure 5D) isolated from Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice displayed a 50% increase in cellular miR-146a levels when compared with those isolated from Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice. We also observed a 3-fold reduction in the number of aortic macrophages in Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice when compared with Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice (Figure 5B). Moreover, resident peritoneal macrophages isolated from Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice displayed lower TNF-α mRNA expression compared with those from Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice (Figure 5C). In peripheral blood, Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice had 2-fold less Ly-6C<sup>high</sup> monocytes compared with Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice (Figure 5E). Intracellular cytokine analysis revealed a 2-fold reduction in the numbers of Ly-6C<sup>high</sup> monocytes producing TNF-α in the spleen of Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice when compared with those of Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice (Figure 5F). Collectively, these results demonstrate that cellular apoE expression, even at low levels seen in HypoE mice, substantially raise miR-146a levels in macrophages and monocytes to reduce chronic inflammation in the setting of hyperlipidemia and thereby suppress atherosclerosis. Systemic Delivery of a miR-146a Mimetic Reduces Macrophage Activation, Ly-6C<sup>high</sup> Monocytosis and Atherosclerosis in the Setting of Hyperlipidemia and ApoE Deficiency

Our in vitro and in vivo findings collectively point to a deficit in miR-146a levels in monocytes and macrophages as a source of increased inflammation and atherosclerosis in mice deficient in apoE expression. We investigated whether correcting such a miR-146a deficit could suppress monocytosis and macrophage activation and thereby atherosclerosis in apoE-deficient mice.
To explore the feasibility of restoring miR-146a levels in monocytes and macrophages in vivo, we systemically delivered a 146a-m or negative control mimetic encapsulated in liposomes (1 nmol/mouse) via tail vein injection twice a week for 2 weeks in miR-146a-deficient (miR-146a<−/−>) mice. This method effectively restored miR-146a expression in Ly-6C<sup>hi</sup> monocytes and macrophages (Online Figure IIA and IIB), confirming an efficient uptake of 146a-m by these cells in vivo. We then explored the benefit of delivering such 146a-m for a period of 6 weeks into Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice fed a normal chow diet. At the time of euthanasia, there was a 3-fold increase in miR-146a expression in peritoneal macrophages isolated from mice injected with the 146a-m than those from mice receiving the negative control mimetic (Figure 6A). Consistent with this finding, the mRNA expression of IRAK1 and TRAF6 were substantially reduced (Figure 6B). Moreover, there was a 50% reduction in TNF-α mRNA levels in resident peritoneal macrophage (Figure 6C), indicating a substantial reduction of NF-κB signaling and inflammation in Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice that received infusions of 146a-m. In addition, as soon as 3 weeks after the 146a-m treatment, Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice also displayed a 30% reduction in the number of blood Ly-6C<sup>hi</sup> monocytes that persisted at the 5-week time point (Figure 6D).

We next examined how such a beneficial reduction in systemic inflammation affected the progression of atherosclerosis in these hyperlipidemic mice. Despite similarly elevated plasma cholesterol levels (Online Figure IIIA), a 6-week period of systemic 146a-m delivery led to a 2-fold reduction in oil-red O positive neutral-lipid surface area (Figure 6E and 6F), as well as macrophage/monocyte monoclonal antibody-2 positive macrophage surface area (Figure 6G and 6H) in the aortic root. In addition, there was a 2-fold reduction in the absolute number of CD11b<sup>+</sup> F4/80<sup>+</sup> macrophages in the aorta of Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice (Figure 6G and 6K). Double immunostaining for macrophage/monocyte monoclonal antibody-2 and Ki-67 (a marker for cell proliferation) in sections of aortic sinuses showed that lesional macrophages in 146a-m–treated mice proliferated at a rate similar to those of control mice (Figure 6H and 6L). Collectively, our findings demonstrate that systemic delivery of 146a-m results in an attenuation of atherosclerosis by suppressing Ly-6C<sup>hi</sup> monocytopoiesis, macrophage activation but not macrophage proliferation in atheroma.

Finally, we assessed the capacity for systemic delivery of a 146a-m to suppress atherosclerosis in hyperlipidemic mice with an intact apoE gene. This was accomplished by using Ldlr<sup>−/−</sup> mice fed a high-fat diet that develop twice the amount of plasma cholesterol as chow fed Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice (Online Figure IIIB). Biweekly infusions of 146a-m for 6 weeks led to a 50% increase of miR-146a in resident peritoneal macrophages of Ldlr<sup>−/−</sup> mice (Figure 7A). This contrasts the 3-fold increase detected in macrophages of 146a-m–treated Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice (Figure 6A). Such modest increase in miR-146a nonetheless reduced the expression of IRAK1, TRAF6, and TNF-α expression in these cells (Figure 7B and 7C). It also led to a 20% reduction in Ly-6C<sup>hi</sup> monocytopoiesis (Figure 7D) and a significant suppression of atherosclerotic plaque formation (Figure 7E and 7G) and macrophage accumulation in the aorta (Figure 7F, 7H, and 7I).

**Figure 5.** Hyperlipidemic HypoE mice expressing reduced apolipoprotein E (apoE) levels display enhanced miR-146a in macrophages and monocytes that suppress their cellular activation. MiR-146a expression in (A) peritoneal macrophages and (D) bone marrow Ly-6C<sup>hi</sup> monocytes isolated from Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> and Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> (HypoE) mice (n=6). B, The number of macrophages in the aortas of Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> and Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice (n=4). C, Tumor necrosis factor (TNF)-α expression in peritoneal macrophages isolated from Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> and Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice (n=6). The number of (E) blood Ly-6C<sup>hi</sup> monocytes and (F) splenic TNF-α-producing Ly-6C<sup>hi</sup> monocytes in Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> and Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice were set arbitrarily to 1 for A, C, and D. Data are shown as the means±SEM. *P<0.05; **P<0.01.

**Discussion**

ApoE is recognized for its unparalleled ability to suppress atherosclerosis.²⁰ Beyond its participation in the removal of atherogenic remnant lipoproteins from plasma,²⁰ apoE exerts a direct influence on myeloid cell activity.²¹ The expression of apoE in the macrophage has long been recognized to suppress atherosclerosis by enhancing the efflux of cellular cholesterol, thereby preventing foam cell formation in the vessel wall.¹² More recent findings have added to the list of apoE’s anti-atherogenic properties that include an ability to suppress myelopoiesis²² and the cellular activation of circulating monocytes in hyperlipidemic mice.¹³ The underlying mechanisms of these protective properties have largely been ascribed to the lipid efflux capacity of apoE both through its cellular expression²³ and by its ability to enhance the cholesterol efflux capacity of plasma high-density lipoprotein.¹⁹ As illustrated in Figure 8, results of our study uncovered a new anti-inflammatory property of apoE. Our findings demonstrate that cellular apoE expression participates to enhance and sustain miR-146a
levels in macrophages to suppress NF-κB–mediated cellular activation in response to acute and chronic inflammatory signaling via TLR receptors, including in a hyperlipidemic environment. We also found that among other leukocytes commonly involved in atherosclerosis, monocytes are the only ones to express appreciable amounts of apoE. Moreover, we noted that apoE is expressed at much higher levels in anti-inflammatory Ly-6C<sup>lo</sup> monocytes that also express far more miR-146a than Ly-6C<sup>hi</sup> monocytes. MicroRNAs have emerged as key regulators of chronic inflammation and atherosclerosis in hyperlipidemic mice. 11,14 In fact, miRNA-146a has been identified as a critical regulator of myeloid cell activation and expansion.10,15 It has been shown to control the balance between pro and anti-inflammatory monocytes by downregulating the expression of the transcription factor RelB that controls the proliferation of Ly-6C<sup>hi</sup> monocytes, which are recognized for their inflammatory24 and atherogenic properties.17 MiR-146a is also recognized for its ability to potently suppress inflammatory challenges by reducing TLR-driven NF-κB signaling in macrophages and in other hematopoietic cells.10 This function is crucial to prevent an immunologic overload and fatal inflammation after a bout of sepsis or LPS injection.15 But the relevance of miR-146a in controlling immunity during prolonged periods of chronic inflammation, such as hyperlipidemia, has not been explored. Therefore, our findings demonstrating reduced miR-146a levels in macrophages and monocytes that lack apoE expression provide new insight to explain the susceptibility to atherosclerosis and sepsis reported in Apoe<sup>−/−</sup> mice.

Figure 6. In vivo delivery of miR-146a (146a-m) mimetics inhibits macrophage inflammatory responses, Ly-6C<sup>hi</sup> monocyteosis and atherosclerosis in hyperlipidemic Apoe<sup>−/−</sup> Ldlr<sup>−/−</sup> mice. Apoe<sup>−/−</sup> Ldlr<sup>−/−</sup> mice (≈10-week-old; n=9–12 per group) receiving a chow diet were intravenously administered miRNA negative control mimetics (NC-m) or 146a-m twice a week for 6 weeks. MiR-146a levels (A), interleukin-1 receptor–associated kinase 1 (IRAK1) and TNF receptor–associated factor 6 (TRAF6; B), as well as tumor necrosis factor (TNF)-α mRNA (C) levels in resident peritoneal macrophages were analyzed by quantitative real-time PCR. The absolute numbers of Ly-6C<sup>hi</sup> monocytes (D) in peripheral blood were determined by flow cytometry before, 3 weeks, and 5 weeks after initiation of the treatment. Representative oil red O staining (E) and macrophage/monocyte monoclonal antibody-2 (MOMA-2) staining (F) of aortic root cross-sections from 146a-m or NC-m treated Apoe<sup>−/−</sup> Ldlr<sup>−/−</sup> mice. Scale bar: 200 μm (E) and 50 μm (F). Quantification of aortic root lesion area (I) and MOMA-2 macrophage area (J). Dots represent values for individual mice; bars are group means. Representative FACS plots (G) and absolute cell number (K) of macrophages in aortas of Apoe<sup>−/−</sup> Ldlr<sup>−/−</sup> mice receiving either 146a-m or NC-m. Live CD45<sup>+</sup> cells were gated. H, Representative images of proliferation marker Ki-67 and MOMA-2 costaining of aortic sinus lesions, arrows denote double positive cells, scale bar, 50 μm; L, Quantification of Ki-67<sup>+</sup> MOMA-2<sup>+</sup> cells in atherosclerotic plaque; data were normalized by MOMA-2 positive area. n=9 to 12 per group (A–D, I, J, and L), n=4 to 6 per group (K). The results of control mice were set arbitrarily to 1 (A–C). Data are shown as the means±SEM. *P<0.05; **P<0.01.
Our findings are unexpected, as miR-146a is known to emerge from NF-κB transcription,10 and Apoe–/– mice have been reported to express increased NF-κB activity,2 which we confirmed in our study. As such, one would legitimately expect to detect increased levels of miR-146a in macrophages isolated from Apoe–/– mice. Contrary to this expectation, we uncovered that cellular apoE expression enables both macrophages and monocytes to upregulate and maintain elevated levels of miR-146a in response to acute and chronic inflammatory challenges. Our findings, therefore, provide provocative new insight to appreciate the influence that apoE can exert on the plasticity of monocytes and macrophages to suppress inflammation and atherosclerosis beyond contributing to enhance cellular lipid efflux.23

The ability of apoE to regulate cellular miRNA levels is not limited to myeloid cells. A study has recently reported that apoE can control miR-145 to suppress smooth muscle cell activation and aortic stiffness.26 Because apoE is known to suppress endothelial activation, it is possible that it could be doing so in part by raising cellular miR-146a levels. Our demonstration of apoE’s capacity to increase miR-146a expression in monocytes and macrophages may constitute a key regulatory mechanism to suppress NF-κB–driven inflammation and atherosclerosis. In addition, because apoE is also expressed by hematopoietic progenitors,23 ongoing studies are aimed at exploring whether it can increase miR-146a to suppress NF-κB signaling and thereby add another level of control over myeloproliferation that has to date been ascribed to its ability to suppress cytokine-driven proliferative signaling by enhancing cellular lipid efflux.25

The physiological relevance of our findings with respect to suppression of chronic inflammation comes from observations of our hypomorphic apoE mouse model of reduced apoE expression that we termed HypoE mice.13 We recently reported that HypoE mice deficient in LDL receptor expression (Apoe<sup>−</sup>−<sup>Ldlr</sup>−/– mice) develop a similar hyperlipidemia as Apoe<sup>−</sup>−<sup>Ldlr</sup>−/– mice, despite significantly lower levels of apoE expression in all tissues, including myeloid cells and hepatocytes.19 A key observation for reduced atherosclerosis in Apoe<sup>−</sup>−<sup>Ldlr</sup>−/– mice was a reduced number of activated circulating Ly-6Chigh monocytes that we ascribed to reduced cellular lipid levels because of enhanced cholesterol efflux capacity of plasma high-density lipoprotein.19 In this study, we uncovered another explanation as Ly-6C<sup>−</sup> high monocytes isolated from these mice were found to express more miR-146a and less TNF-α than those from hyperlipidemic Apoe<sup>−</sup>−<sup>Ldlr</sup>−/– mice. In parallel, peritoneal macrophages isolated from both mouse models demonstrated that a low amount of apoE expression in the HypoE mice was sufficient to raise miR-146a levels and suppress NF-κB–driven cellular activation. An ability for low apoE expression levels to induce an appreciable amount of miR-146a as seen in the HypoE mice was confirmed by our in vitro studies showing that a 25-fold difference in the ectopic expression of apoE in macrophages deficient in apoE results only in a marginal increase in cellular miR-146a levels. Our findings, therefore, underscore the importance of apoE’s capacity to raise cellular miR-146a levels in monocytes and macrophages to suppress NF-κB inflammatory signaling and thereby atherosclerosis. A similar enhancement of miR-146a by apoE in brain astrocytes could play an important role in the protection from NF-κB–driven neurodegenerative disorders.

Cellular miRNA levels are influenced by transcriptional and post-transcriptional events.27 Data from our study demonstrate that cellular apoE expression raises miR-146a levels in monocytes and macrophages by enhancing its transcription. MiR-146a expression is transcriptionally induced by NF-κB
Ldlr–/– mice are less pronounced when given to apoE expression in monocytes and macrophages of Ldlr−/− mice could make them less responsive to infusions of microRNA mimetics. Nonetheless, even a modest 50% increase in macrophage miR-146a levels effectively reduced chronic inflammation and atherosclerosis in hyperlipidemia Ldlr−/− mice. Moreover, a similarly mild elevation in miR-146a levels in macrophages was observed to substantially reduce atherosclerosis in hyperlipidemic HypoE mice (Figure 5). Collectively, our findings support a mild enhancement of miR-146a levels in monocytes and macrophages as a potential therapeutic avenue for chronic inflammatory disorders, including atherosclerosis, while minimizing a compromise of host defense.

In conclusion, our data demonstrate that apoE expression in macrophages and monocytes controls inflammation by enhancing PU.1-dependent miR-146a expression that suppresses inflammatory NF-κB signaling (Figure 8). Our findings provide a new mechanistic link to explain how apoE exerts its anti-inflammatory properties to suppress atherosclerosis beyond reducing plasma lipid levels or enhancing cellular lipid efflux. Our findings also highlight the potential use of targeting the cellular regulation of miR-146a by apoE to promote the resolution of inflammation and atherosclerosis.

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Disclosures

None.

References


In vivo delivery of miR-146a mimetics in hyperlipidemic mice raises miR-146a levels in monocytes and macrophages and effectively suppresses NF-κB–driven inflammation and atherosclerosis in the absence of plasma lipid lowering.

Our understanding of how apoE suppresses atherosclerosis beyond reducing plasma and cellular lipid levels remains incomplete. We found that apoE expression in monocytes and macrophages enhances the production of microRNAs that suppress NF-κB inflammatory signaling, including miR-146a. Although NF-κB activation normally drives miR-146a expression as a natural feedback mechanism, this process is impaired in monocytes and macrophages of apoE deficient mice. The capacity for apoE to increase miR-146a levels was independent of NF-κB activation, but rather was dependent on purine-rich PU-box–binding protein 1, a transcription factor that enhances the expression of miR-146a. Even low levels of cellular apoE expression enhanced miR-146a expression, and this process contributed to suppressed monocyte and macrophage activation and atherosclerosis in hypomorphic apoE mice with hyperlipidemia similar to apoE-deficient mice. Intravenous infusions of miR-146a mimetics rescued the miR-146a paucity in monocytes and macrophages of apoE−/− mice and suppressed NF-κB inflammatory responses and atherosclerosis in the absence of plasma lipid reduction. Such delivery of miR-146a mimetics to hyperlipidemic mice with an intact apoE gene led to a more modest increase in cellular miR-146a levels that nonetheless suppressed atherosclerosis, demonstrating its potential therapeutic use for human atherosclerosis.

What New Information Does This Article Contribute?

- Cellular apoE expression in monocytes and macrophages increases purine-rich PU-box–binding protein 1-dependent expression of miR-146a that suppresses nuclear factor-κB (NF-κB) activation in response to toll-like receptor signaling.
- Sub-physiological apoE expression is sufficient to positively drive monocyte and macrophage heterogeneity by increasing miR-146a levels that suppress inflammation and atherosclerosis in hyperlipidemic mice.
- In vivo delivery of miR-146a mimetics in hyperlipidemic mice raises miR-146a levels in monocytes and macrophages and effectively suppresses NF-κB–driven inflammation and atherosclerosis in the absence of plasma lipid lowering.

What Is Known?

- Apolipoprotein (apoE) can protect against atherosclerosis beyond reducing blood lipid levels.
- Among its pleiotropic properties, apoE has long been recognized to exert regulatory control over cells of the innate and adaptive immune system.
- ApoE is known to protect monocytes and macrophages from lipid-driven activation and contribute to atherosclerosis mainly through its ability to promote cellular lipid efflux.

Novelty and Significance
Apolipoprotein E Enhances MicroRNA-146a in Monocytes and Macrophages to Suppress Nuclear Factor-κB–Driven Inflammation and Atherosclerosis
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SUPPLEMENTAL MATERIAL

Detailed Methods

Mice
All mice were housed and bred in specific pathogen–free conditions in the Animal Research Facility at the San Francisco Veterans Affairs Medical Center. All animal experiments were approved by the Institutional Animal Care and Use Committee at the VA Medical Center. WT C57BL/6, Ldlr<sup>−/−</sup> (B6.129S7-Ldlrtm1Her/J) and Apoe<sup>−/−</sup> (B6.129P2-Apoe<sup>tm1Unc/J</sup>) mice (3-4 weeks old) were purchased from The Jackson Laboratory and fed with a rodent chow diet containing 4.0% fat (Harlan Teklad, 2916), or high fat diet (21% fat, 1.5% cholesterol, Research Diets, D12079B). Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> and Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup> mice, established as previously described, were fed a rodent chow diet of equal nutritional value containing 4.0% fat (Harlan Teklad, 2916) and 9.0% fat (Harlan Teklad, 2919) respectively.

Cells
Bone marrow cells from both the tibias and femurs were harvested from euthanized mice by inserting needles into the bone and washing with RPMI-1640 (UCSF Cell Culture Facility) and filtered through a 70 μM cell strainer (BD Biosciences). Peripheral blood was drawn from anesthetized mice via retro-orbital puncture with heparinized micro-hematocrit capillary tubes (Fisher Scientific). Resident peritoneal macrophages were harvested from mice with 10 ml RPMI 1640 and purified by adherence to tissue culture plastic for 2 hours. Spleens were removed, homogenized gently using plungers, and then filtered through a 70μM cell strainer. Red blood cells were lysed with ACK lysis buffer. Aortic single cells were prepared from Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> and Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup> mice as previously described. In brief, after careful removal of the perivascular fat and cardiac tissue using micro-scissors under a dissecting microscope, single cell suspensions from aortic segments, including the aortic arch and thoracic aorta were prepared by incubation with an enzyme mixture containing 675 U/ml collagenase I, 187.5 U/ml collagenase XI, 90 U/ml hyaluronidase, and 90 U/ml DNase I (all from Sigma-Aldrich) in Hank’s balanced salt solution for 60 min at 37 °C. The resulting single-cell suspensions were washed with Stain Buffer (BD Biosciences) for flow cytometric analysis and sorting or washed with the designed cell culture medium for the in vitro studies.

Cell culture
Bone marrow–derived macrophages (BMDM) from WT and Apoe<sup>−/−</sup> mice were prepared as adherent cultures using recombinant mouse M-CSF (30 ng/ml, Peprotech). For ectopic expression of apoE in Apoe<sup>−/−</sup> BMDMs and Ly-6C<sup>high</sup> monocytes, the cells were cultured in Opti-MEM (Life Technologies) and transiently transfected with mouse Apoe cDNA clone or control expression plasmids (Origene) using Lipofectamine 2000 (Life Technologies) as per the manufacturer’s instructions. For RNA interference experiments, scramble control or apoE siRNA (50 nM, Life Technologies) or PU.1 siRNA (100 nM, GE Dharmacon) were transfected into BMDM or Ly-6C<sup>high</sup> monocytes or BMDM using Lipofectamine RNAiMAX Reagent (Invitrogen) according to the manufacturer’s protocol. At 24 hrs or 48 hrs post transfection, total RNA was extracted and used for qPCR analysis. Target gene knockdown efficiency was validated by qPCR.

Flow cytometry
Fluorescent dye–labeled anti-CD11b (M1/70), anti-F4/80 (BM8), anti-CD19 (1D3), anti-B220 (RA3-6B2), anti-NK1.1 (PK136), anti-Ly-6G (RB6-8C5), anti-Ly-6G (1A8), anti-Ly-6C (AL-21),
anti-CD115 (AFS98), anti-CD11c (HL3), anti-TCR-β (H57-597), and anti-CD3 (145-2C11) antibodies were purchased from BD Biosciences, eBioscience or BioLegend. The gating strategy to identify Ly-6C<sup>high</sup> monocytes (CD11b<sup>+</sup>CD115<sup>high</sup>Ly-6C<sup>high</sup>), Ly-6C<sup>low</sup> monocytes (CD11b<sup>+</sup>CD115<sup>high</sup>Ly-6C<sup>low</sup>), neutrophils (SSC<sup>high</sup>CD11b<sup>+</sup>Ly-6G<sup>+</sup>), CD8α<sup>+</sup> DCs (CD11c<sup>high</sup>CD8α<sup>+</sup>), CD11b<sup>+</sup> DCs (CD11c<sup>high</sup>CD11b<sup>+</sup>), pDCs (CD11c<sup>int</sup>CD11b<sup>+</sup>B220<sup>+</sup>Ly-6C<sup>+</sup>), B cells (B220<sup>+</sup>CD19<sup>+</sup>) and T cells (CD3<sup>T</sup>CD3<sup>C</sup>TCR<sup>-</sup>) are shown in Online Figure VI, V and VI.

Multicolor flow cytometry was performed using standard procedures. Briefly, 1 × 10<sup>6</sup> cells were incubated with anti-CD16/CD32 (2.4G2; UCSF Cell Culture Facility) in 100 μl BD Stain Buffer (DPBS with 0.2% BSA) for 10 min. Without washing, the cells were incubated at 4 °C with combinations of antibodies for 30 min, washed and resuspended in BD Stain Buffer. CountBright Beads (Invitrogen) were added to aortic samples before acquisition to determine the absolute cell numbers.

Intracellular TNF-α staining was performed as previously reported. Briefly, spleen cells were stimulated for 4 h with 50 ng/ml LPS (serotype O55:B5) (Sigma) in the presence of Golgi-plug (BD Biosciences). After stimulation, cells were first stained with cell-surface marker antibodies, fixed and permeabilized with a Cytofix/Cytoperm kit (BD Biosciences), and then stained with APC-conjugated rat anti-mouse TNF-α antibody (BD Biosciences). For intracellular ApoE staining, freshly isolated bone marrow cells or peritoneal cells were first stained with monocyte or macrophage cell-surface marker antibodies, fixed and permeabilized with a Cytofix/Cytoperm kit (BD Biosciences), and then stained with rabbit anti-mouse ApoE antibody (Meridian Life Science, K23100R), followed by a R-PE-conjugated goat anti-rabbit IgG (H+L) antibody (Life Technologies). ApoE antibody was validated for specificity as in Online Figure VII.

For analysis of phosphorylated p65, total blood leukocytes and peritoneal cells were stimulated with 100 ng/mL LPS for 30 min and 15 min respectively, fixed with Cytofix Fixation Buffer, permeabilized with chilled Perm Buffer II or Perm Buffer III (BD Biosciences), and then stained with Alexa Fluor 488 or Alexa Fluor 647-conjugated phospho-NF-κB p65 (Ser536) antibody (93H1; Cell Signaling) and the antibodies specific for surface markers identifying macrophages and monocytes. For PU.1 staining, peritoneal cells or BMDM was first stained with PerCP-Cy5.5-anti-CD11b and FITC-anti-F4/80 antibodies, fixed and permeabilized with a Foxp3/Transcription Factor Staining Buffer Set (eBioscience, 00-5523), and then stained with a Alexa Fluor 647 Conjugated rabbit anti-mouse PU.1 (9G7) mAb (Cell Signaling Technology).

Data were acquired on a LSR II flow cytometer using the FACS Diva software or Accuri C6 cytometer using CFlow Plus software (all instruments and software from BD Biosciences) and analyzed using FlowJo (TreeStar Inc.). All cell sorting was performed on FACS ARIA II or III sorters (Becton Dickinson) at the UCSF flow cytometry core facility.

**Quantitative Real-Time PCR**

Total RNA was extracted from flow cytometry-sorted cells or macrophages using mirVana miRNA Isolation Kit (Life Technologies) according to the manufacturer’s protocol. MiR-146a expression was measured with TaqMan miRNA assays (Applied Biosystems) according to the vendor protocol and normalized by sno202 snRNA levels. To quantitate cellular levels of the NF-κB–related miRNAs, total RNA was polyadenylated and reverse transcribed by using the NCode miRNA first-strand synthesis kit (Invitrogen) according to the manufacturer’s instructions. The resulting cDNA was subjected to qRT-PCR using the NCode universal reverse primer (Invitrogen) in conjunction with a sequence-specific forward primer for individual microRNA. Similarly, sno202 snRNA was quantified using the NCode universal reverse primer and a sno202-specific primer.
To quantitate mRNA, the cDNA was synthesized with total RNA and iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer’s protocol. The expression of genes encoding apoE (Mm01307193_g1), TNF-α (Mm00443260_g1), IL-6 (Mm00446190_m1), IRAK1 (Mm01193538_m1), TRAF6 (Mm00493260_m1), pri-miR-146a (Mm03306349_pri), PU.1 (Mm00488142_m1), β-2 microglobulin (β2m, Mm00437762_m1) and Rn18s (Mm03928990_g1) was assessed by real-time PCR with mouse TaqMan Gene Expression Assays (Life Technologies); results were normalized to expression of the gene encoding β2m or Rn18s and were quantified by the ΔΔCT method as previously described.

**Immunofluorescence and image quantification**

NF-κB p65 immunofluorescence was performed as previously reported. Peritoneal macrophages were seeded onto coverslips in 24-well plates and were stimulated with 100 ng/ml LPS for 15 min. Cells were fixed with 4% paraformaldehyde in PBS (Electron Microscopy Sciences) for 15 min at room temperature. Coverslips were incubated sequentially with 0.1% Triton-X100 (Sigma) (10 min, room temperature), blocking buffer (5% donkey serum in PBS, 60 min, room temperature), NF-κB p65 (D14E12) XP rabbit antibody (D14E12; Cell Signaling) diluted in staining buffer (1% donkey serum in PBS, overnight, 4°C) and Alexa Fluor 568-conjugated donkey anti-rabbit IgG antibody (Invitrogen) diluted in staining buffer (1 hr, room temperature). Nuclei were counterstained with 2 μg/ml of the nuclear stain Hoechst (Invitrogen) for 5 min. Coverslips were mounted on to glass slides (Fisher Scientific) by using SlowFade Gold antifade reagents (Life Technologies). Fluorescence images were captured on a Zeiss Axio Observer Z1 inverted microscope and were analyzed with Cell Scoring Application Module in MetaMorph software (Molecular Devices).

**Plasma cholesterol measurements**

Plasma cholesterol assay was performed using commercial kit (Wako Diagnostics) according to the manufacturer’s instructions.

**Histological quantification of atherosclerosis**

Aortic root sections were stained and quantified as we previously described. Beginning at the base of the aortic root, 75 sections were cut at 10 μm, collected, and arranged in 3 sections per slide. Atherosclerotic lesions in the aortic root were quantified by staining with oil red O to reveal neutral lipids in 15 cross-sections, 20 μm apart starting at the coronary ostium and extending through the base of the aortic valve. Adjacent sections were labeled with a primary rat anti-mouse MOMA-2 antibody (Cedarlane labs, NC) and a rabbit anti-mouse Ki-67 (Abcam), detected with a chicken anti-rat IgG (H+L) antibody and a donkey anti-rabbit IgG (H+L) antibody conjugated with Alexa Fluor 594 and Alexa Fluor 488 (Life Technologies), respectively. Slides were mounted on a Zeiss AxioObserver microscope and images captured with a Retiga-SRV CCD camera equipped with RGB color filter (Qimaging, Surrey, BC, Canada). Oil red O and MOMA-2 Surface areas, and the numbers of Ki-67 positive cells were quantified with Metamorph software (Molecular Devices).

**Statistical analysis**

All statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software). *In vitro* experiment was carried out with three to four independent biological samples. qRT-PCR were performed in two or three technical replicates for each biological sample. Data are presented as means ± SEM. Differences between experimental groups were analyzed for statistical significance by unpaired Student’s t test or by 1-way ANOVA followed by Bonferroni test for the selected pairs. A value of P<0.05 was considered significant.
Online Figure I. MiR-146a levels and activity is reduced in macrophages derived from Apoe<sup>−/−</sup> mice, but not in those derived from Ldlr<sup>−/−</sup> mice

(A) Plasma cholesterol levels in 3~4-week-old WT, Apoe<sup>−/−</sup>, and Ldlr<sup>−/−</sup> mice; (B) qPCR analysis of miR-146a levels in peritoneal macrophages derived from WT, Apoe<sup>−/−</sup>, and Ldlr<sup>−/−</sup> mice; (C) mRNA levels of IRAK1 and TRAF6 in peritoneal macrophages derived from WT, Apoe<sup>−/−</sup>, and Ldlr<sup>−/−</sup> mice were examined by qPCR. n=9~10 for A, n=8 for B and C. *, P<0.05; **, P<0.01.
Online Figure II Validation of delivery of miR-146a mimetics into Ly-6C<sup>high</sup> monocytes and macrophages in vivo

MiR-146<sup>−/−</sup> mice (n=2/group) were intravenously administered miRNA negative control mimetics (NC-m) or miR-146a mimetics (146a-m) twice a week for two weeks. Splenic Ly-6C<sup>high</sup> monocytes (A) and peritoneal macrophages (B) were isolated and analyzed for miR-146a levels by qRT-PCR. The results were compared to WT mice set arbitrarily to 1. N.D., not detectable.
Online Figure III. Systemic delivery of miR-146a mimetics does not alter plasma cholesterol levels in Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice fed a chow diet, or Ldlr<sup>−/−</sup> mice fed a high fat diet. Cohorts of ~10-week old Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice (n=9~12/group) receiving a chow diet or Ldlr<sup>−/−</sup> mice (n=9/group) fed a high fat diet were intravenously administered miRNA negative control mimetics (NC-m) or miR-146a mimetics (146a-m) twice a week. Blood was collected before, 3 weeks and 6 weeks after initiation of the treatment and plasma cholesterol levels were determined by enzymatic assay. A, Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice; B, Ldlr<sup>−/−</sup> mice; Data are shown as the means ± SEM.
Online Figure IV. Gating strategy for the isolation of Ly-6C$^{\text{high}}$, Ly-6C$^{\text{low}}$ monocytes and neutrophils from mouse bone marrow

A, Monocytes were identified as (CD3 B220 NK1.1)$^{-}$ CD11b$^{+}$ CD115$^{\text{high}}$ (CD11c MHCII F4/80)$^{\text{low}}$. Monocytes were then subdivided based on Ly-6C expression. B, Neutrophils were identified as SSC$^{\text{high}}$CD11b$^{+}$Ly6G$^{+}$.
Online Figure V. Gating strategy for the isolation of splenic DC subsets and pDC

CD8α⁺ DC was defined as CD11c⁺CD8α⁺; CD11b⁺ DC was defined as CD11c⁺CD11b⁺.
pDC were identified as CD11c⁻CD11b⁻B220⁺Ly-6C⁺. Int, intermediate.
Online Figure VI. Gating strategy for B cell and T cells in mouse spleen
B cells were defined as B220⁺ CD19⁺; T cells were defined as CD3⁺ TCR-β⁺.
Peritoneal macrophages were isolated from WT mice and incubated with antibodies against CD11b, F4/80, and TCR-β. After fixation and permeabilization, the cells were stained with an isotype antibody (Iso Ab) or an anti-apoE antibody (apoE Ab). Histograms of apoE expression in gated CD11b^+F4/80^{high} macrophages and CD11b^-TCR-β^+ were shown. Ab, antibody.

Supplemental References


