ApoE Suppresses Atherosclerosis by Reducing Lipid Accumulation in Circulating Monocytes and the Expression of Inflammatory Molecules on Monocytes and Vascular Endothelium

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Objective—We investigated atheroprotective properties of apolipoprotein (apo) E beyond its ability to lower plasma cholesterol. We hypothesized that apoE reduces atherosclerosis by decreasing lipid accumulation in circulating monocytes and the inflammatory state of monocytes and the vascular endothelium.

Methods and Results—We developed mice with spontaneous hyperlipidemia with and without plasma apoE. Hypomorphic apoE mice deficient in low-density lipoprotein receptor (Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup>) were compared to Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice. Despite 4-fold more plasma apoE than WT mice, Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup> mice displayed similar plasma cholesterol as Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice but developed 4-fold less atherosclerotic lesions by 5 months of age. The aortic arch of Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup> mice showed decreased endothelial expression of ICAM-1, PECAM-1, and JAM-A. In addition, Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup> mice had less circulating leukocytes and proinflammatory Ly6C<sup>hi</sup> monocytes. These monocytes had decreased neutral lipid content and reduced surface expression of ICAM-1, VLA-4, and L-Selectin. Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup> mice displayed increased levels of apoA1-rich HDL that were potent in promoting cellular cholesterol efflux.

Conclusions—Our findings suggest that apoE reduces atherosclerosis in the setting of hyperlipidemia by increasing plasma apoA1-HDL that likely contribute to reduce intracellular lipid accumulation and thereby the activation of circulating leukocytes and the vascular endothelium. (Arterioscler Thromb Vasc Biol. 2012;32:264-272.)

Key Words: atherosclerosis ■ leukocytes ■ lipoproteins ■ macrophages ■ apolipoprotein E

As a ligand for the receptor-mediated clearance of remnant lipoproteins, apolipoprotein (apo) E is an important modulator of atherosclerosis. The best evidence is provided by the spontaneous hyperlipidemia and atherosclerosis in mice lacking apoE. Beyond its participation in plasma cholesterol lowering, apoE is known to have anti-inflammatory properties. However, because of its ability to reduce plasma cholesterol, investigating mechanisms by which apoE regulates the progression of atherosclerosis in the setting of hyperlipidemia remains challenging. Several approaches that addressed this question succeeded by studying mice expressing low levels of plasma apoE (below the threshold required for plasma cholesterol lowering) derived from macrophages or the adrenal gland. However, unlike many of these murine models, human hyperlipidemia is accompanied by simultaneous accumulation of plasma apoE due to its high affinity for triglyceride-rich lipoproteins. Consequently, mechanisms by which high levels of plasma apoE could serve to reduce atherosclerosis in the setting of hyperlipidemia remain incompletely understood.

To address this issue, we developed mouse models of equal total plasma cholesterol with and without accumulation of plasma apoE. This was achieved by crossing Ldlr<sup>−/−</sup> mice with hypomorphic apoE mice (Apoe<sup>h/h</sup>),<sup>12</sup> and Apoe<sup>−/−</sup> mice to derive Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup> and Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice. We studied these mice to investigate anti-inflammatory properties of apoE on circulating monocytes and the vascular endothelium and its overall effect on atherosclerosis progression.

Previous observations demonstrated that apoE reduced the expression of endothelial adhesion molecules responsible for the recruitment of circulating monocytes to athero-prone regions of the vasculature.<sup>7,13,14</sup> In addition, recent evidence suggests that circulating monocytes can be activated by intracellular lipid accumulation prior to their recruitment to athero-prone regions of the vasculature.<sup>15-17</sup> Whether elevated levels of apoE in hyperlipidemic plasma impact on...
intracellular lipid levels and thereby the inflammatory state of circulating monocytes remains unknown. Thus, we hypothesized that apoE can reduce lipid accumulation in circulating monocytes and the inflammatory state of monocytes and the vascular endothelium and thereby decrease atherosclerosis progression independently of its ability to lower plasma cholesterol. Results of our study highlight novel properties of apoE on the inflammatory state of monocytes and the endothelium in hyperlipidemic mice.

**Methods**

Briefly, Ldlr−/− mice on a C57Bl/6J background (Jackson Laboratories, ME) were bred to hypomorphic apoE mice and to Apoe−/− mice on a C57Bl/6J background (Jackson Laboratories, ME) to create Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> and Apoe<sup>−/−</sup>Ldlr<sup>−/+</sup> mice. Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> and Apoe<sup>−/−</sup>Ldlr<sup>−/+</sup> mice were randomly intercrossed to establish lineages of littermate mice that contained approximately 85% of C57BL/6 and 15% of 129SvJ genetic backgrounds. The San Francisco Veterans Administration Medical Center committee for animal care and welfare approved all procedures. All procedures including blood and tissue collection, plasma lipid and lipoprotein fractionation/isolation with fast protein liquid chromatography (FPLC), and sequential density ultracentrifugation colorimetric assays used to measure plasma lipid levels, SDS-polyacrylamide gel electrophoresis (SDS-PAGE), Western blot, in vitro cholesterol efflux assay, histological and immunofluorescence quantification of atherosclerotic lesions and blood leukocyte analysis by flow cytometry were either performed as described previously or as described in the online Supplemental Data, available online at http://atvb.ahajournals.org.

**Results**

**ApoE Reduces Atherosclerosis Beyond Lowering Plasma Cholesterol Levels**

We previously described the Apoe<sup>−/−</sup> mouse in which a variant form of murine apoE<sup>19</sup> is expressed at 2% to 5% of normal levels.<sup>20</sup> By breeding Apoe<sup>−/−</sup> and Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice to Ldlr<sup>−/−</sup> mice, we generated mice that develop spontaneous hyperlipidemia and atherosclerosis. Interestingly, Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice do so despite accumulating 4-fold more plasma apoE than wild type (WT) mice (Supplemental Figure I). When fed a chow diet containing 5.7% fat, Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice displayed slightly higher plasma cholesterol level (n=10; 584.9–1062 mg/dL) than Apoe<sup>−/−</sup>Ldlr<sup>−/+</sup> mice (n=24; 427.8–854.5 mg/dL). However, feeding Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> and Apoe<sup>−/−</sup>Ldlr<sup>−/+</sup> mice a chow diet of identical nutrient composition but containing 4.2% fat and 9% fat, respectively, brought their plasma cholesterol levels to a similar range. At 14 weeks of age (Supplemental Figure IIA and IIB), both Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> and Apoe<sup>−/−</sup>Ldlr<sup>−/+</sup> mice displayed similar levels of hypercholesterolemia and hypertriglyceridemia. By 20 weeks of age plasma cholesterol levels of Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice reached 634.1±45.23 mg/dL that closely matched those of Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice (628.9±47.48 mg/dL; Figure 1A). Both groups of mice displayed similar plasma triglycerides levels (Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice 189.4±30.08 mg/dL, n=14; Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice 151.0±22.82 mg/dL, n=16 (Figure 1B), body weight, and blood glucose levels (results not shown).

The development of 2 mouse models with similar hypercholesterolemia and hypertriglyceridemia in presence and absence of elevated apoE levels enabled us to investigate how apoE can suppress atherosclerosis beyond lowering plasma cholesterol. Twenty week old Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice developed 1.7-fold less aortic sudanophilic positive lesions than Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice (Figure 1C–1E). In addition, Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice developed 4-fold less aortic root oil red O positive lesions than Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice (53.214±17.102 μm² versus 195.419±...
44,000 μm²; Figure 1F–1H). Beyond being more prominent, lesions of Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice also displayed evidence of necrotic cores containing crystallized cholesterol clefts (black arrows, Figure 1F). As shown in Figure 1I–1K, aortic root lesions of Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup> mice also contained 3-fold less macrophage-positive lesion area than Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice (57,619±17,369 μm² versus 168,307±69,673 μm²). Taken together these results demonstrate that apoE reduces atherosclerosis progression independently of lowering plasma cholesterol.

**ApoE Reduces Endothelial Activation in the Setting of Hyperlipidemia**

ApoE may contribute to reduce macrophage foam cell accumulation by limiting monocyte recruitment to the vascular wall. Thus, we investigated whether apoE reduces atherosclerosis progression in the setting of hyperlipidemia by reducing the expression of adhesion molecules on the endothelium. To test this hypothesis we measured the expression of vascular cell adhesion molecule-1 (VCAM-1), intercellular cell adhesion molecule-1 (ICAM-1), platelet endothelial cell adhesion molecule-1 (PECAM-1), and junctional adhesion molecule-A (JAM-A) on enface preparations of the proximal aorta of 14-week old Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> and Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup> mice (see Supplemental Figure VI, for detailed procedures). We first observed heterogeneous expression of VCAM-1 and ICAM-1 on individual endothelial cells (EC) in both inner and outer curvatures of the aortic arch (Figure 2A and C). VCAM-1, ICAM-1, and JAM-A were expressed on the surface of ECs while PECAM-1, as expected, localized to endothelial cell-cell junctions (Figure 2A, 2C, 2E, and 2G). Unexpectedly, we also observed PECAM-1 redistributing from the cell-cell junctions to the cell surface of EC in aortic arches of Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice (Figure 2E).

The quantification of the mean fluorescence intensity (MFI) for each inflammatory marker (Supplemental Figure IV) revealed significantly lower expression levels of ICAM-1 (1.28-fold), PECAM-1 (4.5-fold), and JAM-A (11-fold) in Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup> mice than in Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice (Figure 2D,F,H). However, no significant difference in the expression level of VCAM-1 was observed between Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> and Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup> mice (Figure 2B). Taken together, our results suggest that plasma apoE contributes to the decreased expression of ICAM-1, PECAM-1, and JAM-A on vascular endothelium in the setting of hyperlipidemia.

**ApoE Reduces Circulating Leukocyte Counts and Monocyte Activation**

A lower count or activation of leukocytes and/or monocytes could also reduce monocyte recruitment to atheroma. To determine the effect of apoE on leukocytes, we analyzed with flow cytometry blood leukocyte count in our mouse models. Despite similar total plasma cholesterol, Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup> mice displayed 30% less blood leukocytes than Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice (Figure 3A). This decrease in blood leukocytes arose from reduced numbers of monocytes, granulocytes, and B cell, but not T cells in Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup> mice (Figure 3B). We also investigated potential differences in monocyte subtypes (defined by Ly6C expression; Figure 3C). Quantification of each monocyte subpopulation revealed 2-fold less Ly6C<sup>high</sup> monocytes in Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup> mice compared to Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice (Figure 3D).

We next measured the expression of cell surface markers of inflammation on circulating monocytes of both mouse models. CD54, CD49d, and CD11a were present on the cell surface of most monocytes (∼100%) in both groups of mice, whereas CD62L, CD11c, and CD31 were present on less than 30% of all monocytes. In addition, we observed a significant
decrease in the level of expression of CD62L (32% less), CD54 (8% less) and CD49d (17% less) in Apoe<sup>h/h</sup> Ldlr<sup>/H11002/H11002</sup> monocytes (Figure 3E) compared to Apoe<sup>/H11002/H11002</sup> Ldlr<sup>/H11002/H11002</sup> mice. Taken together, these results demonstrate for the first time that plasma apoE accumulation contributes to reduce the number of circulating leukocytes, the expansion of Ly6Chigh monocytes, and the overall expression of key adhesion molecules on the surface of monocytes.

**ApoE Decreases Lipid Accumulation in Circulating Monocytes**

In our mouse models neither a high-fat diet nor a difference in plasma lipid levels could explain the reduced count in total leukocytes and Ly6Chigh monocytes and reduced surface expression of adhesion molecules on monocytes. Thus we hypothesized that plasma apoE accumulation contributes to reduce the number of circulating leukocytes, the expansion of Ly6Chigh monocytes, and the overall expression of key adhesion molecules on the surface of monocytes.

Confocal microscopy and flow cytometry analysis of isolated monocytes revealed less intracellular neutral lipid accumulation in Apoe<sup>h/h</sup> Ldlr<sup>/H11002/H11002</sup> monocytes than in Apoe<sup>/H11002/H11002</sup> Ldlr<sup>/H11002/H11002</sup> monocytes (Figure 4A and B). On average, a 16% decrease in neutral lipid accumulation per monocyte was observed in Apoe<sup>h/h</sup> Ldlr<sup>/H11002/H11002</sup> mice compared to Apoe<sup>/H11002/H11002</sup> Ldlr<sup>/H11002/H11002</sup> mice (Figure 4B). In addition, 17% fewer monocytes derived from Apoe<sup>h/h</sup> Ldlr<sup>/H11002/H11002</sup> mice accumulated detectable levels of neutral lipids (Figure 4C). A decrease in neutral lipid accumulation in inflammatory Ly6C<sup>high</sup> (20%) monocyte subtypes from Apoe<sup>h/h</sup> Ldlr<sup>/H11002/H11002</sup> mice was also observed (Figure 4E).

To establish a link between neutral lipid accumulation and an increase in inflammatory monocytes, we correlated the level of intracellular lipid to the surface expression of inflammatory markers. No significant correlations were observed between the expression level of Ly6C and intracellular neutral lipid accumulation (Figure 4F). In contrast, the surface expression of inflammatory markers such as CD62L, CD54, and CD49d positively correlated with neutral lipid accumulation in circulating monocytes. Increased neutral lipid levels correlated with a 50%, 92%, and 77% increased expression of CD62L, CD54, and CD49d, respectively (Figure 4G). By comparison, the expression levels of CD11c, CD31, and CD11a, which showed no difference between Apoe<sup>h/h</sup> Ldlr<sup>/H11002/H11002</sup> mice and Apoe<sup>/H11002/H11002</sup> Ldlr<sup>/H11002/H11002</sup> mice, did not correlate with intracellular neutral lipid levels. Taken together these results demonstrate that plasma Apoe decreases intracellular neutral lipid levels and thereby reduces the overall inflammatory phenotype of monocytes in Apoe<sup>h/h</sup> Ldlr<sup>/H11002/H11002</sup> mice.

**ApoE Modulates Plasma Lipoprotein Composition**

Next, we sought to investigate the potential effects of apoE on plasma lipoprotein composition. We first observed that apoE accumulation in plasma of Apoe<sup>h/h</sup> Ldlr<sup>/H11002/H11002</sup> mice modulated...
the lipoprotein cholesterol profile. The presence of apoE significantly reduced VLDL-cholesterol by 1.4-fold in Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup> mice (Figure 5A). In addition, Western blot analysis of plasma fractionated by FPLC (Figure 5B) revealed that in Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup> mice, apoE associates predominantly with VLDL, IDL, and LDL. In contrast, in the absence of apoE, apoA1 distributed among all lipoproteins classes (Figure 5B). To confirm these results, plasma lipoproteins were fractionated by sequential density ultracentrifugation and analyzed for their lipid and protein content. With this method, we found less VLDL-cholesterol (1.6-fold) but more LDL- (1.7-fold) and HDL-cholesterol (2-fold) content in Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup> mouse plasma than in Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mouse plasma. Lastly, the presence of apoE increased significantly the protein content (1.6-fold) of HDL isolated from Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup> mice compared to that isolated from Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice (Supplemental Table I).

These observations led us to question the potential local influence of apoE on the composition of atheroma. Results of our studies revealed the accumulation of apoE in aortic root lesions of Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice (Figure 5D and F). More specifically, apoA1 immunoreactivity localized mostly to ECs and extracellular matrix surrounding macrophages (Figure 5D and 5F, insets). Lastly, smooth muscle α-actin immunoreactivity was found only in the intimae of Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mouse, revealing the appearance of fibrous caps in the lesions, indicative of a more advanced lesion stage (Figure 5C and 5D, insets).

Enhanced Cholesterol Efflux Capacity of ApoA1-Rich HDL

Finally, we compared the composition and cholesterol efflux capacity of plasma HDL isolated from Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> and Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup> mice by sequential density ultracentrifugation. Consistent with our findings with FPLC fractionated plasma (Figure 5B) we observed that the presence of apoE on the VLDL and LDL fractions of Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup> mice contributed to the redistribution of apoA1 to HDL (Figure 6A and C). We also observed a 1.5-fold enrichment of apoA1 on HDL of Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup> mice compared to that isolated from Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mouse (Figure 6B and 6D). Next, we performed cholesterol efflux experiments with the J7741 mouse macrophage cell line. When normalized for total protein content, HDL isolated from Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup> mouse plasma were 2.3-fold more potent at promoting cholesterol efflux than HDL isolated from Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mouse plasma.

Figure 4. Lipid accumulation in monocytes. Confocal images of monocytes isolated by FACS, (scale bar=10 μm; A). Neutral lipid accumulation in monocytes (B, C), Ly6<sup>C</sup>high and Ly6C<sup>low</sup> subtypes (1-way ANOVA; D and E). Correlations between Lipid Tox and surface marker mean fluorescence intensity (MFI) (F&G). Mean±SEM. *P<0.05, **P<0.01.
The low expression levels of apoE by macrophages of Apoe<sup>h/h</sup> Ldlr<sup>/H11002/</sup> mice also contributed to enhance cholesterol efflux to HDL although to a lower extent. Less than a 5-fold increase in fluorescence intensity (FI) accumulated in media of freshly isolated peritoneal macrophages of Apoe<sup>h/h</sup> Ldlr<sup>/H11002/</sup> mouse incubated with HDL from Apoe<sup>/H11002/</sup> Ldlr<sup>/H11002/</sup> mice (Supplemental Figure VIII) compared to 15-fold increase in FI accumulated in media of J7741 macrophages incubated with HDL from Apoe<sup>h/h</sup> Ldlr<sup>/H11002/</sup> mice (Figure 6).

Taken together, these results indicate that plasma apoE increases HDL-cholesterol and enriches HDL in apoA1, which in turn enhances their potency to promote cellular cholesterol efflux. Thus, apoE-dependent enhancement of cholesterol efflux may at least partly explain the reduced accumulation of lipid within circulating monocytes and lesional macrophages in Apoe<sup>h/h</sup> Ldlr<sup>/H11002/</sup> mice.

**Discussion**

We sought to uncover mechanisms by which apoE can suppress atherosclerosis beyond reducing plasma cholesterol. To this end, we developed a mouse model in which apoE accumulates in the setting of hyperlipidemia. With this model, we showed that plasma apoE contributes to decrease macrophage content in athero-prone regions of the vasculature by reducing lipid accumulation in circulating monocytes and lesional macrophages in Apoe<sup>h/h</sup> Ldlr<sup>/H11002/</sup> mice.

The elevated plasma apoE levels in the setting of hyperlipidemia reduced atherosclerosis in Apoe<sup>h/h</sup> Ldlr<sup>/H11002/</sup> mice. One way by which apoE could reduce macrophage foam cell accumulation within atherosclerotic lesions is by limiting monocyte recruitment. Accordingly, we demonstrated that the presence of apoE in hyperlipidemic mice decreased the
expression of inflammatory molecules on ECs and monocytes that are known to enhance monocyte recruitment to athero-prone regions of the vasculature.22

Evidence that apoE suppressed endothelial activation comes from reduced cell surface levels of ICAM-1, JAM-A, and PECAM-1 on the endothelium of the proximal aorta of Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup> mice. Elevated endothelial expressions of such adhesion molecules are known to be proatherogenic14–23,25 and to mediate the recruitment of monocytes22 to athero-prone regions of the vasculature including the inner curvature of the aortic arch.26 To our knowledge, this is the first report documenting a suppressive effect of apoE on the endothelial expression of JAM-A and PECAM-1. Moreover, in the presence of apoE, we found reduced expression of ICAM-1 but unchanged expression of VCAM-1 unlike results of previous studies.7,13,27

In vitro studies have shown that apoE attenuates cytokine-induced expression of adhesion molecules including VCAM-1 and ICAM-1 by stimulating the endothelial production of nitric oxide.7,27 More recently, Ma et al showed a robust reduction of VCAM-1 and ICAM-1 gene expression in the whole aorta of hyperlipidemic mice expressing subphysiological levels of plasma apoE.8 However, because of other cell types present in an intact aortic arch such as macrophages and smooth muscle cells, which also express VCAM-1 and ICAM-1,28,29 it is difficult to attribute this decreased expression solely to the endothelium. In fact, it is possible that a decrease in macrophages and smooth muscle cells could have contributed to the overall decrease in VCAM-1 and ICAM-1 expression. Alternatively, in our model, the lack of attenuation of VCAM-1 expression in the endothelium of the aortic arch of Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup> mice may have been masked or diluted by the high degree of heterogeneity in the expression of VCAM-1 among ECs (Figure 2A). Accordingly, the analysis of VCAM-1 expression levels on individual ECs of the proximal aorta exposed 2 populations of ECs; 1 with higher and 1 with lower expression levels of VCAM-1. Additionally, this analysis revealed a lower expression level of VCAM-1 among the population of ECs expressing higher levels of VCAM-1 in Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup> compared to those of Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice (data not shown).

Hyperlipidemia and atherosclerosis have been associated with leukocytosis30 and more recently with the increased recruitment of monocytes to athero-prone regions of the vasculature.17,31,32 Studies also demonstrated that an increase in cell survival and proliferation are responsible for hyperlipidemia-induced leukocytosis and monocytosis.16,17,33 A major finding of our study is that elevated plasma apoE levels contribute to reduce leukocyte counts in hyperlipidemic mice. Moreover, the presence of apoE was associated with a reduction in Ly-6C<sup>high</sup> inflammatory monocytes known to be recruited to atherosclerotic lesions.17,32

Enhanced expression of cell adhesion molecules on circulating monocytes contributes to atherosclerosis progression.22 Interestingly, we found that elevated apoE levels in hyperlipidemic plasma decreases the cell surface expression of some known inflammatory molecules17,34 but not that of others16 on circulating monocytes. Studies of Swirski et al have previously shown that Ly6<sup>C</sup>high monocytes consistently express CD62L but not CD11c.17 Accordingly, we observed that in addition to reducing levels of Ly6<sup>C</sup>high monocyte levels, apoE also reduced the cell surface expression of CD62L but not that of CD11c in circulating monocytes. Moreover, we also found that apoE decreases the surface expression of CD49d, a key integrin involved in the arrest and adhesion of monocytes on inflamed endothelium of atherosclerotic lesions.22,34 High cell surface expression of CD54, CD11a, and CD31 on monocytes has also been associated with increased cell adhesion and atherosclerosis22; however, in our hyperlipidemic mouse models, apoE solely decreased the expression of CD54. It is unclear why the presence of apoE reduces the expression of only a select set of adhesion molecules. Nevertheless, this observation constitutes a novel athero-protective property by which plasma apoE suppresses atherosclerosis.

A new concept has emerged demonstrating a direct association between cellular lipid loading and the activation of circulating monocytes.15–17,35 Thus, we propose that elevated plasma apoE levels prevent lipid-induced activation of circulating monocytes in hyperlipidemic mice. Evidence for a role of apoE in this process comes from the lower neutral lipid
content in circulating monocytes of Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup> mice. Additionally, we observed a striking correlation between the level of intracellular neutral lipid and the expression levels of adhesion molecules on circulating monocytes. Cellular lipid accumulation can result from at least 2 different dysfunctional mechanisms: 1) the synthesis of lipids, 2) the uptake of lipoproteins, and 3) the mediation of cholesterol efflux by HDL. ApoE could modulate each one of these processes.

While examining plasma lipoproteins in both mouse models, we observed an important apoE-mediated modulation of plasma lipoprotein composition. ApoA1, which distributed equally among all classes of lipoproteins in Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup> mice, distributed almost exclusively to HDL in Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup> mice. As apoE bound preferentially to VLDL and LDL in Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup> mice, it likely displaced apoA1 from these particles, concentrating it onto HDL. Thus, the presence of apoE likely raised HDL cholesterol and apoA1 levels in the plasma of Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup> mice. Accordingly, HDL-derived from Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup> mice displayed an enhanced ability to promote cellular cholesterol efflux. We also attempted to dissect the direct cellular contribution of apoE from that of its indirect influence on plasma lipoprotein. At first, we assessed the expression level of genes related to cellular lipid metabolism that could potentially be regulated by apoE in isolated macrophages and monocytes (Supplemental Figure VII). The effect of endogenous apoE on the expression level of relevant genes was minimal. We also performed cholesterol efflux experiments with peritoneal macrophages isolated from both Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup> and Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice. Although we cannot rule out the possibility that macrophage-derived apoE, even at low expression level, also contributed to enhance cholesterol efflux in Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup> mice (Supplemental Figure VIII), our results suggest that the apoA1-rich HDL had a greater impact on total cholesterol efflux. In light of previous reports documenting the importance of apoA1 in HDL-mediated suppression of leukocytosis and monocyte activation, apoA1-rich HDL likely contributed to reduce leukocyte counts and the inflammatory state of monocytes observed in Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup> mice. Because of the pleiotropic nature of plasma HDL, we cannot rule out the possibility that higher levels of plasma HDL also contributed to reduce atherosclerosis locally in the arterial wall of Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup> mice by suppressing endothelial activation and foam cell formation. Histological studies of atheromas by confocal microscopy revealed the presence of apoE in intracellular compartments and on the surface of macrophage foam cells. ApoA1 was detected in atheroma of both mouse models. However, as apoA1 resided primarily on HDL in Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup> mice, lesional apoA1 in atheromas of these mice likely contributed to the efflux of cholesterol from foam cells. In contrast, in Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice, lesional apoA1 resided primarily on proatherogenic apoB-containing lipoproteins. These particles were likely less effective at promoting cellular cholesterol efflux and thereby reverse cholesterol transport. Another interesting finding relates to reduced presence of fibrous caps and intimal smooth muscle cells in atheromas of Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup> mice (Figure 5). These results confirm and extend findings of earlier studies that reported a role for apoE in regulating the migration and proliferation of smooth muscle cells. In conclusion, key results of our study highlight apoE’s capacity to reduce lipid-induced leukocyte counts and the inflammatory state of both monocytes and the vascular endothelium. We propose that these new roles of apoE derive in part from its ability to increase apoA1-rich HDL in plasma. Such HDL likely contributed to reduce lipid accumulation in monocytes and thereby their inflammatory state. Thus, these results provide new mechanistic insights to explain how apoE participates to reduce atherosclerosis beyond lowering plasma cholesterol levels.

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**Disclosures**

None.

**References**


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SUPPLEMENTAL MATERIAL

Detailed methods

Materials

All chemicals and reagents were purchased from Sigma-Aldrich, MO, unless otherwise stated.

Animal models

$Ldlr^{−/−}$ mice on a C57Bl/6J background (Jackson Laboratories, ME) were bred to hypomorphic apoE mice expressing an apoE4-like form of murine apoE ($Apoe^{R61^{h/h}}$ mice were approximately 75% C57Bl/6J and 25% 129/SvJ background) $^{1}$ and to $Apoe^{−/−}$ mice on a C57Bl/6J background (Jackson Laboratories, ME) to create $Apoe^{h/h}Ldlr^{−/−}$ and $Apoe^{−/−}Ldlr^{−/−}$ mice. $Apoe^{h/h}Ldlr^{−/−}$ and $Apoe^{−/−}Ldlr^{−/−}$ mice were randomly intercrossed to establish lineages of littermate mice that contained approximately 85% of C57BL/6 and 15% of 129/SvJ genetic backgrounds. $Apoe^{−/−}Ldlr^{−/−}$ and $Apoe^{h/h}Ldlr^{−/−}$ mice were fed a rodent chow diet (Harlan Teklad, Madison, WI) of equal nutritional value containing either 4.2% or 9% of fat (crude oil) respectively. The San Francisco Veterans Administration Medical Center committee for animal care and welfare approved all procedures.

Blood and tissue collection

Blood and tissues were collected from 14 and 20 week-old mice fasted either 4 or 16 hrs. Mice were anesthetized with either isoflurane inhalation or Avertin (Tribromoethanol), and bled by retro-orbital or heart puncture. Mice were perfused via heart puncture with ice-cold PBS (1.5ml/min for 10min) followed with 4% formalin (1.5ml/min for 15min) and glycine in PBS (100mM, 1.5ml/min for 10min) solutions. The formalin-fixed hearts were excised and incubated
in a series of sucrose gradients for cryopreservation, (1 h in 10% and 15% sucrose and overnight in 20% sucrose in PBS). Fixed heart specimens were embedded in Tissue-Tek O.C.T. cryosectioning compound (Sakura Finetek, Japan), and tissue blocks cut into 10-µm-thick sections. Fixed aortic arches were also excised, opened longitudinally to expose the endothelium and processed immediately for immunofluorescence labeling stained with the lipophilic dye Sudan IV for atherosclerosis assessment.

**Plasma lipid and lipoprotein determination**

Plasma lipoproteins were fractionated by fast protein liquid chromatography (FPLC) on a Superose 6 GL 10/30 column (GE Healthcare, NJ; the chromatogram represents an average of results obtained from 3 separate experiments with plasma pooled from 5 mice; mean±sem, two-way ANOVA followed by Bonferonni post-test, *p<0.05) or isolated by sequential density ultracentrifugation as previously described. Briefly, sequential density ultracentrifugation density was performed with plasma pooled from 5 mice. The plasma density was adjusted to 1.021 g/ml with KBr, and plasma centrifuged at 52,000 rpm for 16 h at 8°C in a TLA 100.3 rotor in an Optima TL Ultracentrifuge (Beckman instruments, Fullerton, CA). The top fraction containing VLDL was collected and the bottom fraction adjusted to a density of 1.063 g/ml with KBr and centrifuged for an additional 16 h as described above. The next top fraction containing IDL/LDL was separated from the bottom fraction by adjusting its density to 1.21 g/ml with KBr and a final 16 hr of centrifugation as described above. Finally, the top fraction containing HDL was collected. All lipoprotein preparations were extensively dialyzed against PBS.

Colorimetric assays were used to measure cholesterol, triglycerides and phospholipids levels in plasma, FPLC fractions, and lipoprotein isolated by ultracentrifugation, according to the manufacturer’s instructions (Cholesterol E, L-type TG M, Phospholipids C, Wako, VA). Protein concentrations were determined with the colorimetric Bio-Rad DC Protein Assay (Bio-Rad
Laboratories, Life Science Research, Hercules, CA) according to the manufacturer’s instructions. All colorimetric assays were measured with a VersaMax microplate reader (Molecular Devices Corporation, Sunnyvale, CA). Values were analyzed with either Student t test or two-way ANOVA, followed by Bonferroni post-testing (p<0.05) and expressed as average ± SEM. For FPLC and lipoprotein isolated with ultracentrifugation a minimum of 3 independent experiments were performed, each with plasma pooled from 5 mice.

Plasma, FPLC fractions and lipoprotein preparations were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 4-15% gels and transferred to nitrocellulose or stained with coomassie blue (for each preparation, 30µg of protein were loaded). Western blots were incubated with primary antibodies: rabbit anti-mouse apoE (1:10,000, Meridian Life Science, Inc., ME), followed by detection with a horseradish peroxidase-conjugated anti-rabbit antibody (1:5000 and 1:10,000 respectively, Vector Laboratories Inc., CA). Signals were generated by incubating membranes with chemiluminescent reagent (Pierce, IL) and imaged with an AlphalImager Gel Documentation system (Cell Biosciences, Inc., CA). Band integrated densities were quantified with ImageJ software (U.S. NIH, MD). Coomassie stained gels were imaged and quantified with an Odyssey infrared imaging system (LI-COR Biotechnology, NB). Values were analyzed with t test (p<0.05) and expressed as either fold-increased or average integrated density ± SEM. For each, 3 independent experiments were performed.

**In vitro cholesterol efflux assay**

Immortalized murine macrophages from the J774.1 cell line or freshly isolated peritoneal macrophages from Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup>, Apoe<sup>kh</sup>Ldlr<sup>−/−</sup> and Ldlr<sup>−/−</sup> mice were plated in 12-well tissue culture dishes and allowed to grow to confluence in Dulbecco’s Modified Eagle Medium
(DMEM; Invitrogen) supplemented with 20% fetal bovine serum (FBS, Invitrogen). The cells were first incubated for 36 h with 50µg/ml of human acetylated LDL (acLDL) (BTI, Stoughton, MA) and then for 24 h with 5µg/ml of the fluorescent cholesterol analog 22-NBD-cholesterol (25-[N-[(7-nitro-2-1,3-benzoaziazol-4-yl)methyl]amino]-27-norcholesterol, Avanti Polar Lipids Inc., AL) dissolved in 0.5% ethanol. Medium was changed to DMEM supplemented with 0.3% Bovine serum albumin (BSA) for 24 h to allow equilibration between different cholesterol pools. Equal amounts of HDL (50 µg/ml of protein) isolated from either Apoe–/Ldlr– or Apoe<sup>h/h</sup>Ldlr– mice were added to each well and incubated for 0.5, 1, 2, 4 or 6 h in a CO<sub>2</sub> incubator kept at 37°C. Medias were collected and the accumulated NBD-cholesterol fluorescence measured for each end point with a Gemini EM, fluorescence scanning microplate spectrofluorometer reader (EX-465nm, EM-538nm, Molecular Devices Corporation, Sunnyvale, CA). Measured fluorescence intensity was normalized to basal level. Values were analyzed with two-way ANOVA, followed by Bonferroni post-testing (p<0.05) and expressed as fold-increase ± SEM. Four independent experiments were performed, each in triplicate.

**Histological quantification of atherosclerosis**

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, 20µm 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), detected with a goat anti-rat antibody conjugated with horseradish peroxidase (Vector, CA). All slides were counterstained with hematoxylin. 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). Surface areas covered by oil red O and moma-2 were quantified with Image Pro software (Media Cybernetics, Inc, Bethesda, MD).

**Immunofluorescence characterization of the lesions**

Formalin fixed and glycine quenched cross-sections of the aortic root were permeabilized for 10 min with 0.1% triton-X-100 in PBS. Non-specific labeling was blocked with 10% donkey serum in PBS for 2 h at RT. Sections were then incubated simultaneously with mouse anti-smooth muscle alpha-actin antibody conjugated with Cy3 (1:200, Sigma, MO), rat anti-moma-2 (1:100, Cedarlane, Laboratories Limited, ON, CA), and either rabbit anti-apoE (1:200) or rabbit anti-apoA1 (1:500) antibodies (Meridian Life Science, Inc., ME) in antibody buffer solution (solution of sodium citrate (SSC) supplemented with 2% donkey serum, 0.1% BSA and 0.01% triton-X-100) overnight at 4°C. After three 5 min-washes with 0.01% triton-X-100 SSC, the sections were further incubated in antibody buffer containing Alexa488 conjugated donkey anti-rat and Alexa647 conjugated anti-rabbit secondary antibodies (1:1000; Invitrogen) for 2 h at RT. Following a 3 X 5 min-wash with 0.01% triton-X-100 SSC, the nuclei were stained with Hoechst 33342 (1:10,000; Invitrogen) and slides mounted in SlowFade Gold (Invitrogen). Images of each combination of up to quadruple labels (3 sections, 50 µm apart, starting at the coronary ostium) were acquired with a LSM510 Meta confocal system, 20X, 0.8 NA objectives and optimal pinhole size adjusted for 1 airy disk, (Carl Zeiss Microimaging, Inc, Thornwood, NY) or a Zeiss AxioObserver microscope (as described above) and images acquired and quantified with Metamorph software (Molecular Devices Inc). Controls for cross-reactivity between primary antibody and secondary antibody of a different species were all negative. Clear separation of each channel was also verified by omitting one of the primary antibodies. Observations made in 4-5 mice per group (3 cross-sections per mouse).

**Enface aortic arch immunofluorescence**
Formalin fixed and glycine quenched enface preparation of the aortic arches were permeabilized and immuno-labeled and imaged as described above. Each aortic arch was incubated simultaneously with a goat-anti-VE-cadherin (1:100, Santa Cruz Biotechnology, Inc., CA) and one of the following inflammatory marker; either a rabbit-anti-JAM-A, (1:100, Santa Cruz Biotechnology, Inc.); a rat-anti-CD31 (1:100, BD Pharmingen, CA); a rat-anti-CD106, (1:100, BD Pharmingen); or an Alexa647 conjugated rat-anti-CD54 (1:100, BioLegend, CA), followed by the appropriate combination of secondary antibodies (1:1000; Invitrogen); donkey anti-goat Alexa488 and either donkey anti-rabbit or anti-rat Alexa594. The nuclei were stained with Hoechst 33342 (1:10,000; Invitrogen) and slides mounted in SlowFade Gold (Invitrogen). The same controls as described above were performed. Images were acquired with a LSM510 Meta confocal system, 60X, 1.4 NA objectives and optimal pinhole size adjusted for 1 airy disk, (Carl Zeiss Microimaging, Inc.). A series of 10 to 20 Z sections were collected in order to cover the full thickness of the endothelium layer. The 3D projections were collapsed and displayed with maximum rendering projection prior to analysis and quantification with Image J software. Mean Fluorescence Intensity (MFI) per cell were analyzed with one-way ANOVA followed by Dunn’s multiple comparison test and expressed as mean ± sem arbitrary units (a.u.).

**Blood leukocyte analysis by flow cytometry**

Blood samples were collected by retro-orbital- (14-week old) or by heart- (20-week old) punctures in EDTA and kept at room temperature rocking gently, for the entire staining procedure. Aliquots of 50µl of blood were first incubated for 10-min with FC block (1:500). For neutral lipid stain, blood samples were incubated with LipidTox (1:100, Invitrogen) for 60min. For all other antibodies (PERCP/CY5.5 or APC-CD45, PE-Ly6G, PE-CD3e, APC-CD31 and AF647-CD54, BioLegend; PERCP-Cy5-CD11b, FITC-CD11b, FITC-CD45R/B220, APC-CD62L, APC-CD11c, APC-CD49d and PE-Cy7-CD11a, BD Pharmingen), blood samples were incubated with respective pre-mixed combinations for 20-min. Red cells were then lysed and
samples fixed and diluted with 450µl of BD Red Cell Lysing Solution (BD Pharmingen) for 10min. Samples were analyzed with a C6 Flow Cytometer (Accuri Cytometers Inc., MI), equipped with 2 lasers providing excitation of 488nm and 640nm and 4 colors detection (FL1 530/30nm; FL2 585/40nm, FL3>670nm, and FL4 675/25nm) in addition to linear forward scatter and side scatter detections. The absolute cell counts or cell concentration per µl was determined with the CFlow Plus software (Accuri Cytometers Inc.). A cell population discriminating cell clumps was obtain by gating the cell population for which an increase in forward scatter area signal linearly correlated with an increase in forward scatter height signal (Gate 1, Figure S5). For each sample, 35,000 events in gate P1 were collected. Analyses were performed with FlowJo software (Tree Star Inc., OR).

Blood monocyte isolation

Blood monocytes were isolated with Fluorescence Activated Cell Sorting (FACS). Blood samples obtained from heart puncture were pooled from 2 mice. Leukocytes were first isolated from the uncoagulated blood with a histopaque 1083 and 30min centrifugation at 8 °C. The opaque interface was transferred to a 15ml falcon tube and the cells washed with cold-PBS. The labeling protocol was as follow: the cells were first incubated for 10-min with FC block (1:500), and then incubated for 30min on ice with PerCPCy5-CD45 (BioLegend) and APC-CD115 (eBiosciences, San Diego, CA) antibodies. The labeled cells were washed and re-suspended in Dulbecco’s Modified Eagle Medium (DMEM; Invitrogen) supplemented with 20% fetal bovine serum (FBS, Invitrogen). All samples were sorted with a FACSARia II (BD Pharmingen) and CD45+ CD115+ cells collected. The monocytes population obtained was >98% CD45+CD115+. The isolated cells were either transferred to cover slips for attachment and fixation before fluorescent staining, or processed immediately for RNA extraction.

Laser capture micro-dissection
Heart specimens were excised from sedated mice perfused through the left ventricle with 10ml of ice-cold RNase-free PBS containing RNase inhibitors (ProtectRNA), as described above. The hearts were embedded in Tissue-Tek O.C.T. cryosectioning compound and quickly flash frozen in liquid N₂ cooled-isopentene (-60°C). Sections were serially cut at 20-µm intervals from the base of the aortic sinus and mounted on RNase-free membrane slides 1.0 PEN (Carl Zeiss AG, Germany). Every 5th section was mounted on FisherFrost Plus slides for MOMA-2 staining. For laser capture micro-dissection, sections were fixed in 70% ethanol, counterstained with Hematoxylin QS (Vector Labs) and dehydrated in graded ethanol. Using MOMA-2 stained slides as guide, lesional macrophages were identified and quickly (<25min) dissected using a P.A.L.M. laser capture micro-dissection system (Zeiss). Dissected lesional macrophages obtained from one mouse were pooled for RNA isolation.

**RNA extraction**

RNA was extracted using the Qiagen RNasy Micro kit with a DNase step according to the manufacturer’s instructions (Qiagen Inc., CA). Quantities of RNA were measured with either a NanoDrop™ 2000 (Thermo Scientific, DE) or the Quant-iTM RiboGreen RNA Reagent and kit according to the manufacturer’s instructions (Invitrogen). Quality of RNA was assessed with an Agilent 2100 Bioanalyzer (Agilent, CA). The RNA integrity number (RIN) obtained for isolated monocytes was greater than 9 and for laser captured micro-dissected macrophages greater than 7.5.

**Analysis of gene expression by quantitative real-time RT-PCR**

Quantitative Real-time reverse transcriptase polymerase chain reaction (QRT-PCR) was used to analyze gene expression levels in monocytes isolated by FACS or lesional macrophages micro-dissected from lesions of the aortic root. Gene expression was determined with an ABI Prism 7900 (Applied Biosystems, Foster City, CA) using primer pairs for cybergreen reactions or
Assay-On-Demand for Taqman reactions (Applied Biosystems). cDNA was reverse-transcribed from 3-100ng total RNA (iScript, Bio-Rad Laboratories, Life Sciences Research, Hercules, CA). Two µl of diluted (1:10) cDNA template were used in each qRT-PCR reaction. Each target gene expression was normalized to the average of 2 housekeeping genes (either Cyclophilin A, TATA box binding protein or Beta-2 microglobulin) and calculated according to the $2^{-\Delta\Delta Ct}$ method. All experiments were performed in triplicate.

**Statistical analysis**

Data were analyzed with GraphPad Prism 5 software (GraphPad software inc., La Jolla, CA) using two-tailed Student $t$ tests unless otherwise stated. A difference with a $P \text{ value of } < 0.05$ was considered significant.
Supplemental Figures, Figure Legends

Figure I. Level of plasma apoE and apoA-1 accumulation in 20-week old mice. Plasma apoE levels were analyzed in WT, Ldlr\textsuperscript{−/−} and Apoe\textsuperscript{h/h}Ldlr\textsuperscript{−/−} mice, and plasma apoA-1 levels were analyzed in Apoe\textsuperscript{h/h}Ldlr\textsuperscript{−/−} mice and Apoe\textsuperscript{−/−}Ldlr\textsuperscript{−/−} mice. Plasma was resolved by SDS-PAGE and Western blots were performed with antibodies specific for mouse apoE and apoA-1. Lanes correspond to serial dilutions of 1 µl fasted mouse plasma from a pool of 5 mice.

Figure II. Metabolic parameters of 14-week old mice. Levels of plasma cholesterol (A) and triglycerides (B) from fasted Apoe\textsuperscript{−/−}Ldlr\textsuperscript{−/−} and Apoe\textsuperscript{h/h}Ldlr\textsuperscript{−/−} mice; mean ± sem. Cholesterol distribution in fractionated plasma by fast protein liquid chromatography (FPLC) from fasted Apoe\textsuperscript{−/−}Ldlr\textsuperscript{−/−} and Apoe\textsuperscript{h/h}Ldlr\textsuperscript{−/−} mice (C). The chromatogram represents an average of results obtained from 3 separate experiments with plasma pooled from 5 mice; mean ± sem, 2way ANOVA followed by Bonferonni post-test, no statistical significance observed. Fractions corresponding to the various lipoprotein classes are indicated; very low-density lipoprotein (VLDL), intermediary-density lipoprotein (IDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL).

Figure III. Comparison of atherosclerosis burden between 14-week and 18-week old mice. Representative immuno-fluorescent images of the aortic roots of 14-week (A&B) and 18-week old (C&D) Apoe\textsuperscript{−/−}Ldlr\textsuperscript{−/−} (A&C) and Apoe\textsuperscript{h/h}Ldlr\textsuperscript{−/−} mice (B&D), scale bar=200µm. Cross-sections were labeled with an antibody specific for moma-2, a macrophage marker (red) and Hoechst, a nuclear dye (blue). The auto-fluorescent elastic lamina is shown in green. Quantifications of the lesion area (n=4, *p<0.05; E) and Moma positive area (n=4, *p<0.05; F) are shown.
Figure IV. Quantification of endothelial inflammation in enface preparations of proximal aorta. Proximal aorta were dissected, opened and flattened in order to expose the endothelium of both the inner curvature (athero prone region) and outer curvature (athero resistant region) (A). Triple labeled confocal Z stacks of the entire endothelium layer were collapsed to one plane (3D maximum rendering projection; B). Each channel was then separated and VE-cadherin used to create a mask of regions of interest (ROI=binary skeletonized VE-cadherin signal; C). This mask of ROIs was then applied to the channel image containing the signal to be quantified. The adhesion molecule fluorescence intensity was calculated for each delineated endothelial cell.

Figure V. Gating strategy for flow cytometry analysis. Flow cytometry data were analyzed with FlowJo Software. Representative dot plots and gating strategies are shown. Cell clumps were first excluded by gating the cell population for which an increase in forward scatter area (FSC-A) signal linearly correlated with an increase in forward scatter height (FSC-H) signal (Gate 1 on first dot plot). The cell population from Gate 1 was further gated on CD45 positive cells using a dot plot of side scatter area (SSC) versus CD45 fluorescence intensity (Gate 2 as shown in the second dot plot). The cell populations of Gate 2 were then used to identify monocytes and granulocytes (CD11b versus Ly6G logarithmic fluorescence intensity dot plot) or B cells and T cells (B220 versus CD3ε logarithmic fluorescence intensity dot plot).

Figure VI. Laser capture micro-dissection of lesional macrophages. Lesional macrophage foam cells were dissected from cross-sections of the aortic root by making use of a guide slide stained with the macrophage marker moma-2 shown (A). Cross-sections prior (B) and after (C) micro-dissection, scale bar=200µm. Fold difference in the expression levels of the macrophage marker CD68 (n= 7 mice/group, Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice versus Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup> mice) and the smooth muscle cell marker α-actin (n= 5 mice/group, Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice versus Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup> mice)
normalized to the housekeeping gene cyclophilin from samples of RNA extracted from micro-dissected

**Figure VII. Effect of apoE on gene expression in circulating monocytes and lesional macrophages.** Expression of genes associated with lipid mobilization (A&D), cholesterol influx (B&E) and cholesterol efflux (C&F) pathways in circulating monocytes (A-C) and lesional macrophages (D-F) prepared by laser capture micro-dissection from 20-week old *Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup>, Apoeh<sup>h/h</sup>Ldlr<sup>−/−</sup> and wild type (WT) mice. Adaptin-2 complex subunit alpha-2 (AP2A2), Actin, alpha-4 (ACTN4), Prosaposin (PSAP), Lipoprotein lipase (LPL), Cathepsin L (CTSL), Cystatin C (CYSC), Transferrin (TRF), Low density lipoprotein receptor-related protein 1 (LRP1), Scavenger receptor B (CD36), Sterol-responsive element-binding protein-2 (SREBP2), 3-hydroxy-3-methylglutaryl-coenzyme A (HMGCoA), Liver X receptor alpha (LXRα), Liver X receptor beta (LXRβ), ATP-binding cassette transporter A-1 (ABCA1), ATP-binding cassette transporter G-1 (ABCG1); mean ± sem are shown, *p<0.05.

**Figure VIII. Effect of macrophage-derived apoE on cholesterol Efflux.** Representative images of primary culture of peritoneal macrophages from *Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup>, Apoeh<sup>h/h</sup>Ldlr<sup>−/−</sup>, and *Ldlr<sup>−/−</sup> mice labeled with anti-apoE (green) and Hoechst (blue; scale bar=50µm; A). Primary culture of peritoneal macrophages were loaded with NBD-cholesterol and the FI accumulation in media quantified after 24hrs of passive efflux with and without treatment with cAMP (B) and following incubation with HDL (100µg protein/ml) from *Apoeh<sup>h/h</sup>Ldlr<sup>−/−</sup> mice for 0.5, 1, 2 and 4 hours (E), n=3, mean ± sem, two-way ANOVA, **p<0.01 and ***p<0.001 between macrophages of *Ldlr<sup>−/−</sup> and *Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice; $p<0.05 between macrophages of *Ldlr<sup>−/−</sup> and *Apoeh<sup>h/h</sup>Ldlr<sup>−/−</sup> mice; #p<0.05 between macrophages of *Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> and *Apoeh<sup>h/h</sup>Ldlr<sup>−/−</sup> mice.
### Supplemental Table

Table I. Percent of lipids and proteins content in lipoprotein subclasses separated by sequential density ultracentrifugation.

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<th>Proteins</th>
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<td>E&lt;sup&gt;h/h&lt;/sup&gt;</td>
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<tr>
<td><strong>VLDL</strong></td>
<td>Mean ± SEM</td>
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<tr>
<td><strong>HDL</strong></td>
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*<i>p<0.05</i> between Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> (E<sup>−/−</sup>) and Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup> (E<sup>h/h</sup>), n=3.
Supplemental Results and Discussion

ApoE and lipid homeostasis in circulating monocytes and lesional macrophages

Collectively, the influx, biosynthesis, and efflux of lipids can influence cellular lipid levels in monocytes and lesional macrophages. Because apoE is known to participate in these pathways in macrophages and as apoE is also expressed by circulating monocytes, we wondered whether apoE reduces cellular lipid levels by regulating the expression of genes associated with these pathways. As such, we measured the relative expression of genes associated with pathways responsible for lipid mobilization with quantitative real time PCR in monocytes isolated with FACS and lesional macrophages isolated with laser capture micro-dissection (Figure VI) from WT, Apoe<sup>h/h</sup>Ldl<sup>−/−</sup> and Apoe<sup>−/−</sup>Ldl<sup>−/−</sup> mice (Figure VII). As shown in Figure VII A, C, D & F, no significant difference in the expression of genes associated with lipid mobilization and cholesterol efflux pathways were found in circulating monocytes and lesional macrophages of Apoe<sup>h/h</sup>Ldl<sup>−/−</sup> and Apoe<sup>−/−</sup>Ldl<sup>−/−</sup> mice. However, there was a significant increase in the expression of ABCA-1 and ABCG-1 in monocytes isolated from Apoe<sup>−/−</sup>Ldl<sup>−/−</sup> mice when compared to WT mice (*p<0.05, n=2-5; Figure VII C). Nevertheless, a few differences in the expression of genes associated with the cholesterol influx and biosynthesis pathway were detected in both monocytes and lesional macrophages between Apoe<sup>h/h</sup>Ldl<sup>−/−</sup> and Apoe<sup>−/−</sup>Ldl<sup>−/−</sup> mice. Monocytes isolated from Apoe<sup>h/h</sup>Ldl<sup>−/−</sup> mice expressed 1.6-fold less of the transcription factor SREBP-2 than monocytes isolated from Apoe<sup>−/−</sup>Ldl<sup>−/−</sup> and WT mice. The expression of LRP-1 was also found to be 1.8-fold higher in monocytes isolated from both Apoe<sup>h/h</sup>Ldl<sup>−/−</sup> and Apoe<sup>−/−</sup>Ldl<sup>−/−</sup> mice than in those from WT mice (*p<0.05, n=2-5; Figure VII B). In contrast, while no difference in the expression level of SREBP-2 in lesional macrophages was found between the two groups of mice, lesional macrophages isolated from Apoe<sup>h/h</sup>Ldl<sup>−/−</sup> mice expressed 1.7-fold less of the scavenger receptor A (CD36) than those from Apoe<sup>−/−</sup>Ldl<sup>−/−</sup> mice (Figure VII E). Overall,
although the small differences in the expression of genes associated with cholesterol influx and biosynthesis may contribute favorably to differential lipid homeostasis, they are likely insufficient to explain the magnitude of reduced lipid accumulation in both monocytes and lesional macrophages present in Apoe<sup>h/h</sup> Ldlr<sup>−/−</sup> mice.

SREBP-2 is a transcription factor activated by low intracellular sterol levels and best known to up-regulate genes involved in cholesterol biosynthesis and uptake. Conversely, we observed lower expression of SREBP-2 in monocytes of Apoe<sup>h/h</sup> Ldlr<sup>−/−</sup> mice. Thus, the role of SREBP-2 in our mouse models is unclear at the moment. As for the scavenger receptor CD36, its reduced expression in lesional macrophages of Apoe<sup>h/h</sup> Ldlr<sup>−/−</sup> mice may have contributed to reduce lipid ingestion and foam cell formation. Alternatively, lower CD36 expression could have lessen the trapping of macrophages within the arterial wall and consequently reduced atherosclerosis in Apoe<sup>h/h</sup> Ldlr<sup>−/−</sup> mice.

ApoE has recently been identified as a key component in the macrophage sterol response network (MSRN) that is up-regulated in response to cellular lipid loading. However, in our hyperlipidemic mouse models, low levels of apoE expression did not impact on the regulation of the MSRN in either monocytes or lesional macrophages at least at the mRNA level. However, since the expression level of genes associated with the MSRN in monocytes derived from both hyperlipidemic mouse models was comparable to that of WT mouse monocytes, the impact of apoE expression on the MSRN may have occurred at the level of protein expression. Conversely, we found an increase in the expression of a few genes in monocytes isolated from both models of hyperlipidemia relative to those isolated from WT mice. This included a twofold increase in the expression of LRP1. Although it is not clear why monocytes would upregulate the expression of the LRP1 in the setting of
hyperlipidemia, it could be related to the absence of LDL receptor expression in our models. We also detected similarly robust increases in the expression of both ABCA-1 and ABCG-1 in monocytes derived from both Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup> mice and Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup> mice as compared to those from WT mice. These results suggest that monocytes isolated from both mouse models of hyperlipidemia were equally stimulated to up-regulate lipid transporters, as the expression of both LXRα and LXRβ were slightly but not significantly increased in either strain of mice, perhaps as a consequence of excess sterol absorption.

Collectively, these findings point to the possibility that factors other than intracellular apoE expression contributed significantly to reduce neutral lipid levels in both monocytes and lesional macrophages of the Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup> mice. Since circulating monocytes from Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup> mice expressed low levels of apoE (results not shown), an extracellular source of apoE could have played a greater role in modulating lipid accumulation within both monocytes and lesional macrophages.

**Supplemental References**


Figure I
Figure V
Figure VIII