Inducible Apoe Gene Repair in Hypomorphic ApoE Mice Deficient in the Low-Density Lipoprotein Receptor Promotes Atheroma Stabilization with a Human-Like Lipoprotein Profile

Delphine Eberlé, Fu Sang Luk, Roy Y. Kim, Victor R. Olivas, Nikit Kumar, Jessica M. Posada, Kang Li, Nathalie Gaudreault, Joseph H. Rapp, Robert L. Raffai

Objective—To study atherosclerosis regression in mice after plasma lipid reduction to moderately elevated apolipoprotein B (apoB)–lipoprotein levels.

Approach and Results—Chow-fed hypomorphic ApoE mice deficient in low-density lipoprotein receptor expression (Apoeh/Ldlr–/–Mx1-cre mice) develop hyperlipidemia and atherosclerosis. These mice were studied before and after inducible cre-mediated Apoe gene repair. By 1 week, induced mice displayed a 2-fold reduction in plasma cholesterol and triglyceride levels and a decrease in the non–high-density lipoprotein:high-density lipoprotein-cholesterol ratio from 87%:13% to 60%:40%. This halted atherosclerotic lesion growth and promoted macrophage loss and accumulation of thick collagen fibers for up to 8 weeks. Concomitantly, blood Ly-6C^hi monocytes were decreased by 2-fold but lesional macrophage apoptosis was unchanged. The expression of several genes involved in extracellular matrix remodeling and cell migration was changed in lesional macrophages 1 week after Apoe gene repair. However, mRNA levels of numerous genes involved in cholesterol efflux and inflammation were not significantly changed at this time point.

Conclusions—Restoring apoE expression in Apoeh/Ldlr–/–Mx1-cre mice resulted in lesion stabilization in the context of a human-like ratio of non–high-density lipoprotein:high-density lipoprotein-cholesterol. Our data suggest that macrophage loss derived in part from reduced blood Ly-6C^hi monocytes levels and genetic reprogramming of lesional macrophages. (Arterioscler Thromb Vasc Biol. 2013;33:1759-1767.)

Key Words: apolipoprotein E ● atherosclerosis ● macrophage ● monocyte

Atherosclerosis-related cardiovascular disease remains a leading cause of death worldwide.1 Atherosclerotic lesions develop in part from the infiltration of circulating monocyte-derived macrophages within the arterial intima in response to an accumulation of apolipoprotein B (apoB)–containing lipoproteins secondary to hyperlipidemia.2 Advanced and unstable lesions can rupture causing thrombosis, acute cardiovascular events, and death.3 Thus, plaque stabilization and regression are important therapeutic goals. Promising results were recently obtained with intensive lipid-lowering therapies using high doses of statins in humans.4 Because mice respond poorly to the cholesterol-lowering effect of statins,4 multiple strategies, ranging from aortic arch transplant5 to transgene induction6–11 often performed in combination with diet change, have been developed to trigger significant plasma lipid lowering. Studies of some of these models showed that during plaque remodeling, lesional macrophages displayed increased expression of genes associated with cholesterol efflux and anti-inflammatory pathways.10,12,13 It was also shown that macrophages egressed from lesions by a C-C chemokine receptor type 7 (CCR7)–dependent mechanism.10,12,13 However, in another study using a different mouse model,15 reduced macrophage content during plaque

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Regression resulted from decreased monocyte recruitment, not from their egress. Such discordant results highlight the challenge of determining whether these findings are applicable to a general process that would be expected to occur in humans. Thus, investigating mechanisms of plaque regression in alternative mouse models is important to clarify the steps of this clinically relevant process.

Most studies of atherosclerosis regression have investigated the process in the context of plasmas containing high levels of high-density lipoproteins (HDLs) and very low levels of plasma apoB lipoproteins (or non-HDL lipoproteins). In this study, we sought to develop a mouse model in which hyperlipidemia could be reduced to moderately elevated levels of plasma apoB lipoproteins. To achieve this goal, we made use of our previously described hypomorphic ApoE mice (Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup>Mx1-cre) also referred to as HypoE mice. The hypomorphic ApoE allele results in low apoE expression levels (5%–10% of wild-type levels). HypoE mice fed a chow diet display a lipoprotein profile dominated by HDLs similar to wild-type mice. However, HypoE mice are susceptible to diet-induced hyperlipidemia and atherosclerosis that can be reversed by switching them to a chow diet alone and by inducible cre-mediated repair of the hypomorphic ApoE allele.

More recently, we have reported that HypoE mice deficient in apoE expression, reduced hepatic apoE immunoreactivity was mainly detected in hepatic sinusoids at baseline, hepatocytes displayed increased intracellular staining after plpC injections (Figure 2A), indicating that plpC treatment effectively repaired the hypomorphic ApoE allele in Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup>Mx1-cre mice.

One week after plpC treatment, both total plasma cholesterol and triglyceride levels were decreased by 2.1-fold to 337±27 and 182±15 mg/dL, respectively (Figure 1A and 1B). Plasma lipid lowering persisted to the 4-week time point in plpC-treated mice, whereas no change was observed in control saline-treated mice (Figure 1A and 1B). The reduction of plasma lipid levels observed after repair of the hypomorphic ApoE allele was associated with significant changes in lipoprotein levels and composition. One week after injection, plpC-treated mice displayed a 3.8-fold decrease in apoB lipoprotein-cholesterol levels (Table I in the online-only Data Supplement). Specifically, levels of very-low density lipoprotein (VLDL)- and intermediate density lipoprotein/LDL-cholesterol were reduced by 5.7- and 2.6-fold, respectively (Figure 1D; Table I in the online-only Data Supplement). In addition, plasma apoB48 and apoB100 levels were reduced by 3.7- and 1.2-fold, respectively (Figure 1C). Concomitantly, plpC-induced mice showed an increase in plasma HDL-cholesterol and apoA1 levels by 1.4- and 1.6-fold, respectively (Figure 1D and 1C; Table I in the online-only Data Supplement). Although we observed a change in the distribution of apoE among plasma lipoproteins before and after intervention, total plasma apoE levels in plasma remained unchanged (Figures 1C, 2C, and 2D). The repair of the hypomorphic ApoE allele was characterized by an enrichment of apoE in large HDLs that eluted in fractions 18 to 20 after fast protein liquid chromatography (Figure 2C) and were found within the 1.02- to 1.06-g/mL lipoprotein density fraction after density ultracentrifugation (Figure 2D). Overall, within 1 week of plpC treatment the drop in cholesterol among apoB lipoproteins and rise among HDLs led to a decrease of the non-HDL:LDL cholesterol ratio from 7 (87%:13%) to 1.5 (60%:40%; Table I in the online-only Data Supplement; Figure 1). These changes in plasma lipids and lipoprotein levels persisted 4 weeks after plpC treatment, and were independent of any change in body weight or blood glucose level (Figure III in the online-only Data Supplement) and occurred in mice continuously fed a chow diet.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
Inducible Repair of the Hypomorphic ApoE Allele in Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup>Mx1-cre Mice Restores Hepatic apoE Expression, Reduces Hyperlipidemia, and Improves the Ratio of Non-HDL:LDL-cholesterol

By 20 weeks of age, chow-fed Apoe<sup>h/h</sup>Ldlr<sup>−/−</sup>Mx1-cre mice displayed severe hypercholesterolemia and hypertriglyceridemia (719±28 and 390±28 mg/dL, respectively, at baseline; Figure 1A and 1B). Inducible repair of the hypomorphic ApoE allele in these mice was induced by 2 intraperitoneal injections of polynosinic-polycytidylic ribonucleic acid (plpC) 1 day apart. One week after plpC treatment, ApoE mRNA expression levels increased by 5-fold in the liver, whereas they were not changed after saline injections (Figure 2B). Although apoE immunoreactivity was mainly detected in hepatic sinusoids at baseline, hepatocytes displayed increased intracellular staining after plpC injections (Figure 2A), indicating that...
size from a parallel group of mice injected with saline were included as a control for lesion progression. As shown in Figure 3A and 3C, saline treatment resulted in increased aortic root lesion area compared with baseline levels (+60% and +90% for the 20- and 27-week-old groups respectively). However, lesion areas of pIpC-treated mice were similar in size at all time points compared with their respective baseline levels (Figure 3A and 3C) indicating that atherosclerosis progression had been halted. To address whether lesion sizes decreased after a longer period, 20-week-old mice were followed for up to 8 weeks after intervention. Lesion sizes were not different from baseline (Figure IV in the online-only Data Supplement). Although the progression of aortic root atherosclerosis in both 20- and 27-week-old mice was halted, we did not observe a significant change in the neutral lipid content of atheroma (Figure 3B and 3C; Figure IVB in the online-only Data Supplement). High-power magnification of oil red O–stained sections revealed neutral lipid deposits that appeared to be localized both within and outside of residual foam cells (Figure V in the online-only Data Supplement).
Neointimal lesion area was highly correlated with the area positive for oil red O ($R^2=0.92; P<0.0001$ and $R^2=0.48; P<0.0001$ for both 20- and 27-week-old groups, respectively), indicating that neutral lipids accumulated in the atheroma proportionally to lesion size and independently of the treatment.

**Figure 3.** Area (A) and neutral lipid content (B) of aortic root lesions from 20- and 27-week-old group (n=6–16 for each time point). Representative oil red O-stained sections (C; scale bar, 200 μm). Aortic arch lipid levels (D; n=8 per group, cholesterol esters (CE), free cholesterol (FC) mean±SEM; *P<0.05, **P<0.01 vs baseline by Dunnett test post-ANOVA). b indicates baseline; and plpC, polyinosinic-polycytidylic ribonucleic acid.

**Figure 4.** Macrophage (A) and collagen (B) content of aortic root lesions from 20- and 27-week-old group (n=6–16 for each time point; mean±SEM; *P<0.05, **P<0.01, and ***P<0.001 vs baseline by Dunnett test post-ANOVA). Representative cross-sections (C) with MOMA-2+ macrophages (green), smooth muscle cells (red), nuclei (blue), and sirius-red-stained collagen viewed under bright or polarized light (scale bar, 100 μm). b indicates baseline; and plpC, polyinosinic-polycytidylic ribonucleic acid.
To further characterize atherosclerotic lesions, aortic arches were excised from 20-week-old mice before and 4 weeks after treatment. Aortic lipids were extracted and quantified. Although the content of cholesterol esters and free cholesterol tended to increase in aortic arches of saline-treated mice (P=0.09), there was no change in plpC-treated mice compared with baseline levels (Figure 3D). Overall, these results indicate that repairing the hypomorphic Apoe allele in Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup>Mx1-cre mice halted atherosclerosis progression in the aortic arch and aortic root but did not lead to plaque regression or an overall loss of arterial lipids.

**Apoe Gene Repair Leads to Macrophage Removal and Accumulation of Thick Collagen Fibers in Atheroma of Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup>Mx1-cre Mice**

We next investigated lesion composition. As shown in Figure 4A and 4C, the content of MOMA-2–positive macrophages in atheroma was significantly reduced 4 weeks after plpC injection compared with baseline (−18% and −37% for the 20- and 27-week-old groups, respectively), whereas no change was observed in atheroma of saline-treated mice. The loss in lesional macrophages was accompanied by a marked increase in collagen content (+70% and +75% for the 20- and 27-week-old groups, respectively; Figure 4B). Lesional macrophage loss and collagen accumulation persisted for up to 8 weeks after Apoe gene repair in 20-week-old mice and in fact was accentuated at this time point (−32% and +135%, respectively; Figure IV, D, and F in the online-only Data Supplement; Table IV in the online-only Data Supplement). Collagen content was also increased in lesions of saline-treated mice (+48% and +59% for the 20- and 27-week-old groups, respectively; Figure 4B). However, atheromas of plpC- and saline-treated mice clearly differed in their relative proportion of intimal collagen and macrophages with a higher ratio of collagen to macrophages in plpC-treated mice compared with saline control mice (1.7-fold; P=0.06 and 1.7-fold; P=0.05 for 20- and 27-week-old groups, respectively). The distribution and type of collagen that accumulated also differed between plpC- and saline-treated mice (Figure 4C; Figure VI in the online-only Data Supplement). Lesions of plpC-treated mice were characterized by densely packed, thick, and strongly birefringent yellow-red type I collagen fibers within macrophage-depleted area that are recognized for their rigidity. In contrast, lesions of saline control mice contained more diffuse networks of green type III collagen fibers surrounding foam cells, known for their extensibility. The proportion of type I to type III collagen was quantitatively increased in lesions of plpC-treated mice compared with those of saline control mice in the 27-week-old group (Figure VIB in the online-only Data Supplement). No difference was observed in the 20-week-old group (Figure VIA in the online-only Data Supplement), suggesting a lesion stage-specific effect. Overall, these quantitative and qualitative analyses indicate that Apoe gene repair in Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup>Mx1-cre mice was associated with increased markers of lesion stability.

**Apoe Gene Repair Does Not Impact Lesional Macrophage Apoptosis in Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup>Mx1-cre Mice**

To address whether increased macrophage apoptosis contributed to plaque macrophage loss, we quantified cells positive for MOMA-2, Hoescht, and TUNEL (Figure 5A) in lesions of mice at baseline and 4 weeks after plpC or saline treatment in both 20- and 27-week-old cohorts. Apoptotic macrophages were observed within lesions of all groups at an expected low frequency (Figure 5B). As shown in Figure 5C, the frequency of apoptotic macrophages (calculated by the number of apoptotic macrophage per square millimeter of MOMA-2 area) was unchanged between baseline and plpC-injected mice in both 20- and 27-week-old cohorts.

**APOE-Dependent Plasma Lipid Lowering in Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup>Mx1-cre Mice Changes Gene Expression in Lesional Macrophages**

Prior studies of murine models of plasma lipid lowering and atheroma remodeling identified changes in the expression of genes involved in cholesterol efflux, macrophage polarity (M1/M2), and egress from lesion. To gain insight into the processes that led to lesion remodeling and macrophage loss in our model, we investigated molecular changes in macrophages isolated by laser capture microdissection from aortic root lesions. Lesional macrophages at baseline were compared with those isolated 1 week after treatment. No significant changes were observed in mRNA levels of genes related to cholesterol efflux (Lxrα, Lxrβ, Abca1, Abcg1; Figure 5D), M1 inflammatory (Tnfα, Mcp1), or M2 anti-inflammatory responses (Mgl1, Tgfβ; Figure 5E). In addition, the expression of Ccr7, previously implicated in macrophage egress during plaque remodeling, showed a trend for an increase (P=0.27) but was not significantly changed at the mRNA level at this time point (Figure 5E).

To further investigate possible changes in gene expression in lesional macrophages, we used a real-time polymerase chain reaction array containing 600 genes related to cardiovascular diseases (OpenArray, Inc). We identified 23 genes that displayed significantly altered mRNA levels 1 week after treatment (Figure 5F; Table II in the online-only Data Supplement). Among these, several genes were associated with extracellular matrix remodeling and cell migration, including matrix metalloproteinase-2 and -19 (Mmp2 by 0.6-fold, Mmp19 by 4.1-fold), cell adhesion regulator (Spg7 by 2.2-fold), chondroitin sulfate proteoglycan 2/Versican (Vcan by 1.6-fold), and integrin-β2 (Itgb2 by 1.27-fold; Figure 5F; Table II in the online-only Data Supplement). Of note, mRNA expression levels of proapoptotic (Bax, Caspase-3 and 6) and antiapoptotic (Bcl2 and Bcl-xL) genes, present on the OpenArray, were unchanged 1 week after lipid lowering (Table III in the online-only Data Supplement).

**Apoe Gene Repair Decreases Blood Ly-6C<sup>high</sup> Monocytes in Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup>Mx1-cre Mice**

Because blood monocyte recruitment into plaques is strongly affected by monocyte levels in the circulation, we examined whether restoration of apoE expression affected blood monocyte levels in the 27-week-old group of mice. As shown in
A 30% decrease in the number of total circulating monocytes was observed 2 weeks after pIpC-induced Apoe reexpression, whereas there was no reduction in saline-treated mice. The reduction in blood monocytes derived mainly from a 64% decrease in the number of Ly-6Chigh monocyte subsets (Figure 6B), whereas the number of Ly-6C low monocytes did not change significantly (Figure 6C). There was no detectable change in the numbers of Ly-6Chigh and Ly-6C low monocyte subsets in the saline-treated mice (Figure 6B and 6C).

Discussion

Plaque stabilization and regression are important therapeutic goals to prevent atherosclerosis-related cardiovascular disease. However, the underlying cellular and molecular mechanisms that govern these processes remain incompletely understood. In the present study, we studied a mouse model of reversible hyperlipidemia, in which atheroma remodeling occurs in the context of mildly elevated plasma apoB lipoprotein levels and a human-like ratio of non-HDL:HDL-cholesterol. Apoe h/hLdlr–/–Mx1-cre mice develop spontaneous hyperlipidemia and atherosclerosis on a chow diet. Here, we show that restoring normal apoE expression in chow-fed Apoe h/hLdlr–/–Mx1-cre mice rapidly decreased plasma lipids and apoB lipoprotein levels, consistent with the notion that hepatic apoE production is required for the clearance of remnant lipoproteins in the absence of the LDL receptor. Inducible Apoe gene repair in these mice also lead to a rise in plasma HDL-cholesterol and apoA1 levels accompanied by enrichment in large apoE-rich HDLs supporting the proposed role of apoE in promoting HDL expansion and remodeling. The resulting non-HDL:HDL-cholesterol ratio decreased from 7 (87%:13%) to 1.5 (60%:40%). These values differed from many other mouse models of atherosclerosis regression.
and remodeling as they remained within the range of a lipo-protein ratio seen in humans.

In this model, we showed that atherosclerosis progression was halted and that atheroma adopted features of stabilization for up to 8 weeks after Apoe gene repair. However, there was no evidence of lesion regression. Several studies in humans and mice including our prior studies of HypoE mice suggested that a drastic reduction in circulating apoB lipoproteins was a key permissive component for lesion regression. Thus, it is possible that levels of apoB lipoprotein remained too high in our model (>200 mg/dL) for lesion regression to occur. Of interest, lesion regression was not observed in other mouse models that display drastic reductions of apoB lipoprotein levels such as in the Reversa mouse or in Ldlr<sup>−/−</sup> mice switched from a high-fat diet to a chow diet. Conversely, lesion regression was reported in mice, despite very high level of non-HDL-cholesterol levels. Thus, taken together, our study and those of others suggest that lesion regression is influenced by several diverse parameters and not solely by reductions of plasma lipid levels.

Further, we showed that Apoe gene repair induced lesion stabilization as evidenced by an accumulation of thick collagen fibers and macrophage loss. Atheroma stabilization was reported in mice with high plasma apoB lipoproteins in which HDL levels were elevated by apoA1 overexpression. Thus, the increase in HDL particles observed after Apoe gene repair likely contributed to lesion remodeling in our model. Changes in plaque composition occurred without a change in neutral lipid content as assessed by oil red O staining. The amount of cholesterol esters and free cholesterol in the aorta was also quantitatively unchanged after intervention. Consistent with our findings of no net lipid loss from atheroma, we did not detect changes in the expression of genes involved in cholesterol efflux (Lxrα, Lxrβ, Abca1, and Abcg1) in lesional macrophages 1 week after lipid lowering. Overall, our results indicate that lesional macrophage loss can occur independently of net arterial lipid loss and of increased cholesterol efflux capacity. Van Craeyveld et al recently reported plaque remodeling with a reduction in macrophage content without net neutral lipid loss in Ldlr<sup>−/−</sup> mice after LDL receptor gene repair. However, in that study, gene expression was assessed in whole lesions from aortic roots and brachiocephalic arteries, not specifically in lesional macrophages. In our study, mRNA levels of Lxrα and Ccr7 were not significantly changed in lesional macrophages 1 week after Apoe gene repair. Nevertheless, Ccr7 expression might have been differently expressed at an earlier or later time point as previously observed in studies of the aortic arch transplant model and the Reversa mouse showed that macrophage emigration to regional and systemic lymph nodes participated in their removal during lesion regression and remodeling. This process relied on liver X receptor-dependent upregulation of the chemokine receptor CCR7 in macrophages which confers dendritic cell-like features. Van Craeyveld et al recently failed to detect increased Ccr7 expression during plaque remodeling in Ldlr<sup>−/−</sup> mice 12 weeks after LDL receptor transgene induction. However, in that study, gene expression was assessed in whole lesions from aortic roots and brachiocephalic arteries, not specifically in lesional macrophages. In our study, mRNA levels of Lxrα and Ccr7 were not significantly changed in lesional macrophages 1 week after Apoe gene repair. Nevertheless, Ccr7 expression might have been differently expressed at an earlier or later time point as previously observed in studies of the aortic arch transplant model and the Reversa mouse.

Levels of monocytes in the circulation strongly affect the rate of their infiltration in the vessel wall where they transform into plaque macrophages. We found that the Ly-6C<sup>high</sup> monocyte subset was reduced after Apoe gene repair. Ly-6C<sup>high</sup> monocytes are known to preferentially accumulate in growing atheroma in hyperlipidemic mice. The frequency of apoptotic lesional macrophages was unchanged after apoE restoration. So, it is possible that decreased Ly-6C<sup>high</sup> monocyte recruitment, coupled with a stable rate of macrophage apoptosis, led to reduced lesional macrophage content in our model. Interestingly, Murphy et al recently demonstrated that cell-derived apoE inhibits hematopoietic stem cell proliferation and monocytopoiesis in hyperlipidemic mice. Thus, the inducible cre-mediated repair of the hypomorphic Apoe allele in Apoe<sup>−/−</sup>Ldlr<sup>−/−</sup>Mx1-cre mice may have affected hematopoietic stem cell proliferation and monocytopoiesis. The potential importance of blood monocytes in modulating lesional macrophage loss during plaque remodeling was highlighted in 2 recent studies. First, a decrease in blood monocyte levels was observed in Ldlr<sup>−/−</sup> mice fed an atherogenic diet followed by a switch to a low-fat diet. Second, Potteaux et al recently demonstrated that suppressed monocyte activation and recruitment coupled with a stable rate of apoptosis accounted for macrophage loss in regressing lesions of high-fat diet–fed Apoe<sup>−/−</sup> mice treated with apoE-encoding adenoviral vectors. Monocytopoiesis is an independent risk factor for atherosclerosis progression in humans. Thus, our findings and those of others underscore the potential impact of modulating circulating monocyte levels, phenotypes, and recruitment to achieve atheroma remodeling.

Loss of macrophages from lesions could also result from their egress. Several studies of the aortic arch transplant model and the Reversa mouse showed that macrophage emigration to regional and systemic lymph nodes participated in their removal during lesion regression and remodeling. This process relied on liver X receptor-dependent upregulation of the chemokine receptor CCR7 in macrophages which confers dendritic cell-like features. Van Craeyveld et al recently failed to detect increased Ccr7 expression during plaque remodeling in Ldlr<sup>−/−</sup> mice 12 weeks after LDL receptor transgene induction. However, in that study, gene expression was assessed in whole lesions from aortic roots and brachiocephalic arteries, not specifically in lesional macrophages. In our study, mRNA levels of Lxrα and Ccr7 were not significantly changed in lesional macrophages 1 week after Apoe gene repair. Nevertheless, Ccr7 expression might have been differently expressed at an earlier or later time point as previously observed in studies of the aortic arch transplant model and the Reversa mouse.

Other factors could also account for the differences between our results and earlier studies. The lesion stage at which the intervention was applied or the withdrawal of the atherogenic diet to induce lipid lowering may both have influenced lesional gene expression independently of lipid lowering. Potteaux et al recently demonstrated that lesional macrophage loss during lesion remodeling was independent of a role of CCR7-induced egress in the high-fat diet–fed Apoe<sup>−/−</sup> mouse after apoE was reintroduced by liver-directed gene transfer. Collectively, these observations suggest that plaque macrophage loss could occur independently of the upregulation of the CCR7 pathway in some mouse models and not in others. Additional studies will be required to conclude on the role of CCR7-dependent or independent egress in our model.

Atherosclerotic lesions ultimately progress toward unstable phenotypes characterized by the upregulation of matrix metalloproteinases, such as Mmp2, involved in the breakdown of collagen. Interestingly, in our model, the expression of Mmp2 was decreased in lesional macrophages 1 week after...
Apoe gene repair, supporting the concept that lesional macrophages undergo an active genetic reprogramming that likely contributes to collagen accumulation and lesion stabilization. Increases in the expression of genes involved in cell mobility, such as Mmp19 or Vasp, or cell adhesion, such as Vcan or Itgb2, were also observed after treatment, suggesting that residual macrophages participate in a dynamic restructuration of the lesion that could involve cellular migration within or out of atheroma. Future studies will explore in more detail the role of individual candidate genes in atherosclerosis remodeling.

In conclusion, the inducible repair of the hypomorphic Apoe allele in chow-fed Apoe<sup>6</sup>Ldlr<sup>−/−</sup>Mx1-cre mice resulted in plasma lipid lowering with mildly elevated apoB lipoproteins and a human-like ratio of non-HDL:HDL-cholesterol. These changes led to stabilization of atherosclerotic lesions, characterized by macrophage loss and accumulation of thick collagen fibers. Our data support that plaque macrophage loss during lesion stabilization and remodeling derives in part from reduced blood Ly-6C<sup>high</sup> monocyte levels and genetic reprogramming of lesional macrophages.

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Disclosures
None.

References
We introduce hypomorphic apolipoprotein E (HypoE) mice deficient in low-density lipoprotein receptor expression (ApoE<sup>h/h</sup>LDLr<sup>–/–</sup> Mx1-Cre mice), as a model of spontaneous and reversible hyperlipidemia in which to investigate the biology of atheroma remodeling, stabilization, and regression in mice continually fed a chow diet that display a human-like lipoprotein profile. Restoring normal apoE gene expression in these mice by inducible gene repair reduces hyperlipidemia to moderately elevated plasma low-density lipoprotein while increasing apoA1 and apoE in high-density lipoprotein. These changes suppress the expansion of activated blood monocytes and promote atheroma stabilization within 1 week, characterized by lesional macrophage loss, collagen accumulation but no net loss of arterial lipid. Persistence of atheroma stabilization for up to 8 weeks, despite mild hyperlipidemia, suggests the possibility that apoE caused epigenetic alterations that prevented a relapse of atherosclerosis. Future studies of the model may lead to new avenues for the therapeutic treatment of atherosclerosis beyond promoting arterial lipid loss.
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SUPPLEMENT MATERIAL

Supplemental Figure Legends

Figure I. Blood monocyte analysis was performed by flow cytometry using an Accuri C6 Flow cytometer with 4 color detection, forward scatter (FSC-A, FSC-H), side scatter (SSC-A) and volume. Cellular debris and aggregates were first gated out, and then cells (leukocytes, neutrophils and monocytes) were identified using CD45-APC, Ly6G-PE, CD11b-PerCPCy5.5 and Ly-6C-FITC as indicated on the representative dot plots and gating strategies.

Figure II. Lesional macrophages were dissected from aortic root sections using a guide slide stained with the macrophage marker MOMA-2 (in red) (A). Sections prior to (B) and after (C) laser captured microdissection. Collection tube with dissected material (D).

Figure III. Body weight (A) and blood glucose (B) in 4h-fasted Apoe<sup>h/h</sup> Ldr<sup>−/−</sup> Mx1-cre mice after plpC or saline injections (n=7-23 per group). Mean±SEM. ***p<0.001 versus baseline, b is for baseline.

Figure IV. Area (A), neutral lipid content (B), macrophage (C) and collagen (D) content of aortic root lesions from 20-week old mice at baseline and 8 weeks after apoE induction (n=9-16 for each time point). Mean±SEM, ***p<0.001 versus baseline by t-test. The 27-week old baseline group presented in Figure 3 and 4 was used as an approximate control progression group. Values and statistical comparison are summarized in Table IV. (E) Representative Oil Red O-stained sections (Scale bar= 200µm) with (F) magnified lesion area showing MOMA-2<sup>+</sup> macrophages (green), smooth muscle cells (red), nuclei (blue), and sirius-red-stained type I (yellow-red) and type III (green) collagen viewed under bright or polarized light.

Figure V. High magnification representative pictures of Oil Red O-stained sections from 27-week old Apoe<sup>h/h</sup> Ldr<sup>−/−</sup> Mx1-cre mice at baseline and 4 weeks after plpC or saline injections (Scale bar=200µm).

Figure VI. Relative proportions of collagen type I and type III fibers in lesions of (A) 20-week old and (B) 27-week old Apoe<sup>h/h</sup> Ldr<sup>−/−</sup> Mx1-cre mice at baseline and 4 weeks after plpC or saline injections. (n=6-16 for each time point). Mean±SEM, ***p<0.05 by t-test.
## Supplemental Tables

**Table I.** Cholesterol distribution among plasma lipoprotein fractions before and 1 week after apoE gene repair

<table>
<thead>
<tr>
<th>Lipoprotein Fraction</th>
<th>Cholesterol (mg/dl)</th>
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<td></td>
<td>Baseline 1w</td>
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<tr>
<td>VLDL</td>
<td>268 ± 14 47 ± 6 **</td>
<td>34.3 ± 0.5 13.4 ± 0.5 **</td>
</tr>
<tr>
<td>IDL/LDL</td>
<td>416 ± 29 162 ± 13 *</td>
<td>53.1 ± 0.1 47.2 ± 0.9 *</td>
</tr>
<tr>
<td>HDL</td>
<td>99 ± 11 136 ± 14 NS</td>
<td>12.6 ± 0.5 39.4 ± 0.4 **</td>
</tr>
<tr>
<td>Non-HDL/HDL ratio</td>
<td>7.0 ± 0.3 1.5 ± 0.02 **</td>
<td></td>
</tr>
</tbody>
</table>

Plasma was fractionated by FPLC. Each lipoprotein was defined as the sum of the following fractions: VLDL (5-9), LDL/IDL (10-19) and HDL (20-28). Data are average from 2 separate pools of 4 mice each.

* p<0.05, ** p<0.01 from t-test between baseline versus 1 week
Table II. Effects on mRNA levels in lesional macrophages from Apoe<sup>h/h</sup> Ldr<sup>+/</sup> Mx1-cre<sup>+</sup> mice 1 week after apoE gene repair

<table>
<thead>
<tr>
<th>Assay ID</th>
<th>Assay RefSeq</th>
<th>Assay Description</th>
<th>Baseline (n=6)</th>
<th>1w (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean±SD</td>
<td>Mean±SD</td>
</tr>
<tr>
<td>Increased mRNA levels</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mmp19</td>
<td>NM_021412</td>
<td>Matrix metallopeptidase 19</td>
<td>1.00±0.49</td>
<td>4.13 ± 2.15</td>
</tr>
<tr>
<td>Ccl5</td>
<td>NM_013653</td>
<td>Chemokine (C-C motif) ligand 5 / RANTES</td>
<td>1.00±0.75</td>
<td>3.25 ± 0.99</td>
</tr>
<tr>
<td>Slc2a1</td>
<td>NM_011400</td>
<td>Solute carrier family 2 member 1 / Glut1</td>
<td>1.00±0.91</td>
<td>2.49 ± 0.60</td>
</tr>
<tr>
<td>Birc3</td>
<td>NM_007464</td>
<td>Apoptosis inhibitor 1</td>
<td>1.00±0.35</td>
<td>2.26 ± 0.72</td>
</tr>
<tr>
<td>Spg7</td>
<td>NM_153176</td>
<td>Cell adhesion regulator</td>
<td>1.00±0.36</td>
<td>2.21 ± 0.85</td>
</tr>
<tr>
<td>Alox5ap</td>
<td>NM_009663</td>
<td>Arachidonate 5 lipoxygenase activating protein</td>
<td>1.00±0.22</td>
<td>1.92 ± 0.33</td>
</tr>
<tr>
<td>Bcl2a1a</td>
<td>NM_009742</td>
<td>B-cell leukemia</td>
<td>1.00±0.23</td>
<td>1.84 ± 0.59</td>
</tr>
<tr>
<td>Tnip2</td>
<td>NM_139064</td>
<td>A20 binding inhibitor of NF-kappaB activation-2</td>
<td>1.00±0.05</td>
<td>1.67 ± 0.33</td>
</tr>
<tr>
<td>Vcan</td>
<td>NM_001081249</td>
<td>Chondroitin sulfate proteoglycan 2/Versican</td>
<td>1.00±0.17</td>
<td>1.60 ± 0.37</td>
</tr>
<tr>
<td>Cav1</td>
<td>NM_007616</td>
<td>Caveolin 1</td>
<td>1.00±0.26</td>
<td>1.49 ± 0.21</td>
</tr>
<tr>
<td>Cyba</td>
<td>NM_007806</td>
<td>Cytochrome b-245, alpha polypeptide</td>
<td>1.00±0.20</td>
<td>1.40 ± 0.31</td>
</tr>
<tr>
<td>Cd44</td>
<td>NM_009851</td>
<td>CD44 antigen</td>
<td>1.00±0.21</td>
<td>1.38 ± 0.24</td>
</tr>
<tr>
<td>Tnfrsf1a</td>
<td>NM_011609</td>
<td>TNF receptor alpha chain</td>
<td>1.00±0.21</td>
<td>1.34 ± 0.26</td>
</tr>
<tr>
<td>Atox1</td>
<td>NM_009720</td>
<td>Antioxidant protein 1</td>
<td>1.00±0.11</td>
<td>1.32 ± 0.21</td>
</tr>
<tr>
<td>Vasp</td>
<td>NM_009499</td>
<td>Vasodilator-stimulated phosphoprotein</td>
<td>1.00±0.10</td>
<td>1.29 ± 0.22</td>
</tr>
<tr>
<td>Itgb2</td>
<td>NM_008404</td>
<td>Integrin beta 2</td>
<td>1.00±0.15</td>
<td>1.27 ± 0.12</td>
</tr>
<tr>
<td>Nisch</td>
<td>NM_022656</td>
<td>Imidazoline receptor 1-1-like protein</td>
<td>1.00±0.12</td>
<td>1.21 ± 0.17</td>
</tr>
</tbody>
</table>
**Decreased mRNA levels**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession</th>
<th>Description</th>
<th>Baseline</th>
<th>1 Week</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pld3</td>
<td>NM_011116</td>
<td>Phospholipase D family, member 3</td>
<td>1.00</td>
<td>0.81</td>
<td>0.045</td>
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<tr>
<td>Nr3c1</td>
<td>NM_008173</td>
<td>Glucocorticoid receptor 1</td>
<td>1.00</td>
<td>0.73</td>
<td>0.017</td>
</tr>
<tr>
<td>Mmp2</td>
<td>NM_008610</td>
<td>72kD gelatinase</td>
<td>1.00</td>
<td>0.63</td>
<td>0.007</td>
</tr>
<tr>
<td>Gstt1</td>
<td>NM_008185</td>
<td>Glutathione S-transferase, theta 1</td>
<td>1.00</td>
<td>0.47</td>
<td>0.007</td>
</tr>
<tr>
<td>Esr1</td>
<td>NM_007956</td>
<td>Estrogen receptor 1 (alpha)</td>
<td>1.00</td>
<td>0.41</td>
<td>0.023</td>
</tr>
<tr>
<td>Bmp2</td>
<td>NM_007553</td>
<td>Bone morphogenetic protein 2</td>
<td>1.00</td>
<td>0.18</td>
<td>0.010</td>
</tr>
</tbody>
</table>

Relative mRNA levels in lesional macrophages from *Apoeb<sup>eh</sup> Ldr<sup>−/−</sup> Mxl-cre* mice before (baseline) and 1 week after induced-apoE gene repair (n=6 mice per group). Mean±SD. P value from t-test between baseline versus 1 week.
Table III. Expression levels of pro- and anti-apoptotic genes in lesional macrophages from Apoe^{h/h} Ldr^{-/-} Mx1-cre^+ mice 1 week after apoE gene repair

<table>
<thead>
<tr>
<th>Assay ID</th>
<th>Assay RefSeq</th>
<th>Assay Description</th>
<th>Mean</th>
<th>SD</th>
<th>Mean</th>
<th>SD</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pro-apoptotic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bax</td>
<td>NM_007527</td>
<td>Bcl2-associated X protein</td>
<td>1.00</td>
<td>0.29</td>
<td>1.27</td>
<td>0.21</td>
<td>0.124</td>
</tr>
<tr>
<td>Casp3</td>
<td>NM_009810</td>
<td>Caspase 3</td>
<td>1.00</td>
<td>0.74</td>
<td>0.61</td>
<td>0.37</td>
<td>0.319</td>
</tr>
<tr>
<td>Casp6</td>
<td>NM_009811</td>
<td>Caspase 6</td>
<td>1.00</td>
<td>0.24</td>
<td>1.04</td>
<td>0.27</td>
<td>0.812</td>
</tr>
<tr>
<td><strong>Anti-apoptotic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bcl2</td>
<td>NM_009741</td>
<td>B-cell leukemia</td>
<td>1.00</td>
<td>0.24</td>
<td>1.10</td>
<td>0.37</td>
<td>0.628</td>
</tr>
<tr>
<td>Bcl-xl</td>
<td>NM_009743</td>
<td>Bcl2l1 B-cell leukemia</td>
<td>1.00</td>
<td>0.44</td>
<td>0.93</td>
<td>0.38</td>
<td>0.798</td>
</tr>
</tbody>
</table>

Relative mRNA levels in lesional macrophages from Apoe^{h/h} Ldr^{-/-} Mx1-cre mice before (baseline) and 1 week after induced-apoE gene repair (n=6 mice per group). Mean±SD. P value from t-test between baseline versus 1 week.
Table IV. Aortic root lesion size and composition in 20-week old mice at baseline and 8 weeks after apoE induction, and in 27-week old mice at baseline.

<table>
<thead>
<tr>
<th></th>
<th>20-week old group Baseline (n=16)</th>
<th>20-week old group 8w plpC (n=9)</th>
<th>27-week old group Baseline (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lesion size (μm²)</td>
<td>96522 ± 12603</td>
<td>82253 ± 14509</td>
<td>185475 ± 185475 (p=0.01)</td>
</tr>
<tr>
<td>Macrophage content (% Lesion)</td>
<td>70.0 ± 2.7</td>
<td>47.2 ± 3.0</td>
<td>57.5 ± 3.3 (p=0.03)</td>
</tr>
<tr>
<td>Collagen content (% Lesion)</td>
<td>26.4 ± 2.4</td>
<td>62.1 ± 1.4</td>
<td>35.1 ± 2.7 (p&lt;0.0001)</td>
</tr>
<tr>
<td>Ratio Collagen/ Macrophage</td>
<td>0.40 ± 0.05</td>
<td>1.42 ± 0.10</td>
<td>0.62 ± 0.05 (p&lt;0.0001)</td>
</tr>
</tbody>
</table>

Data are presented as Mean ± SEM. The 27-week old baseline group was used as an approximate control progression group for 20-week old mice 8 weeks after plpC treatment (8w plpC). P values are from t-test comparing 20-week old group, 8w plpC versus 27-week old group, Baseline.
Figure II

A  Guide slide
B  Slide pre-capture
C  Slide post-capture
D  Collection tube
Figure III

A

Body weight (g)

B

Blood glucose (mg/dl)

plpC

saline

b 1w 2w 4w 2w 4w
Figure IV

A

Lesion area (x10^6 μm^2)

B

Oil Red O (%) Lesion

C

Moma (% Lesion)

D

Collagen (% Lesion)

E

baseline

8w plpC

F

MOMA-2

α-SMA

Sirius brightfield

Sirius polarized
Figure V

baseline

4w plpC

4w saline
Figure VI

A

20-week old

Collagen type (%)

0 50 100 150

b 4w 4w

plpC saline

B

27-week old

Collagen type (%)

0 50 100 150

b 4w 4w

plpC saline

* Type III

Type I
MATERIAL AND METHODS

Mice and Experimental Design
Male Apoe<sup>h/h</sup>Ldl<sup>–/–</sup>Mx1-cre mice were weaned at 21 days and fed a chow diet containing 9% fat (w/w) (#2919, Harland Teklad, Madison, WI) throughout the study. Briefly, these mice, who have a 85% C57BL/6 and 15% 129/SvJ genetic background, are characterized by their reduced Apoe expression due to the presence of a flox-flanked neomycin cassette in the third intron of the mouse Apoe gene<sup>2</sup>. They spontaneously develop hyperlipidemia and atherosclerosis on a low fat diet<sup>1</sup>. Separate groups of mice were euthanized either at a baseline time point (20 or 27 weeks old), or 1, 2, 4 or 8 weeks after treatment. One group of mice received 2 intra-peritoneal injections of polyinosinic-polycytidylic ribonucleic acid (pIpC) (Sigma, MO) (250 µg per mouse) at 48h interval to induce the Mx1-cre transgene<sup>3</sup> and repair of the hypomorphic Apoe allele<sup>2</sup>. The second group of mice received normal saline injections as a control group. The numbers of mice for each study group is indicated in parenthesis: 20-week old group baseline (16), 1w pIpC (7), 2w pIpC (7), 4w pIpC (14), 8w pIpC (9) and 4w saline (8); and 27-week old group baseline (8), 4w pIpC (12) and 4w saline (6). All mice were housed in a barrier facility with a 12h light/12h dark cycle. All animal work was approved by the Institutional Animal Care and Use Committee of the San Francisco Veterans Affairs Medical Center.

Metabolic Parameters, Plasma Lipids and Lipoprotein Fractionation
Body weight, blood glucose, plasma lipids and lipoproteins were monitored in 4h-fasted mice. Blood glucose was measured with a Contour glucometer (Bayer, Mishawaka, IN) using a drop of blood collected by tail incision. Mice were anesthetized with isoflurane (3 to 5% in oxygen for 4 min) and plasma was obtained from blood drawn from the retro-orbital sinus. For lipoprotein analysis, plasma was pooled from four mice and fractionated by fast performance liquid chromatography (FPLC) on a Superose 6 GL 10/30 column (GE Healthcare, NJ) or by sequential density ultracentrifugation using KBr gradient (d<1.006 (VLDL), 1.006<d<1.020 (IDL), 1.020<d<1.063 (LDL), 1.063<d<1.021 (HDL)) as previously described<sup>4</sup>. Briefly, the plasma density was first adjusted to 1.006 g/ml with PBS and centrifuged at 52 000 rpm for 24 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 was adjusted to a density of 1.020 g/ml with KBr and centrifuged for an additional 24 h as described above to isolate IDL. This step was repeated twice to isolate LDL and HDL at the previously mentioned density. Lipoproteins isolated by density ultracentrifugation were dialyzed against phosphate buffered saline (PBS) and analyzed by 4-15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue staining. Lipid levels in plasma and lipoprotein fractions were measured by colorimetric assays according to the manufacturer’s instructions (Total and free Cholesterol E, L-type TG M, Phospholipids C, Wako, VA) adapted for a VersaMax microplate reader (Molecular Devices, Corporation, Sunnyvale, CA).

Western Blot Quantitation of ApoE, ApoA1 and ApoB
Plasma and FPLC fractions were resolved by SDS-PAGE, transferred to nitrocellulose membranes (Bio-Rad Laboratories, CA) and subjected to western blotting with primary antibodies directed against mouse apoE (1/2500 to 1/10 000),<sup>5</sup> apoA1 (1/30 000, Meridian Life Science, Inc., ME) and apoB (1/10 000).<sup>5</sup> Signal was quantified using either IRDye680LT-conjugated anti-rabbit antibody and the Odyssey system (LI-COR Biosciences, NE) or horseradish peroxidase-conjugated anti-rabbit antibody (Vector Laboratories Inc., CA), chemiluminescent reagent (Pierce, IL) and Alphalmager Gel Documentation system (Cell Biosciences, Inc., CA).

Analysis of Atherosclerotic Lesion Size and Composition
Mice were fasted for 4h (at 20 weeks old) or overnight (at 27 weeks old), anesthetized by intra-peritoneal injection of tribromoethanol (Avertin, 250 mg/kg of body weight) and sacrificed by heart blood puncture followed by perfusion with 10ml of ice-cold PBS containing RNase inhibitor (Sigma, MO). Hearts were embedded in tissue freezing medium (OCT) and flash frozen in an isopentane bath for histology or laser-
capture dissection studies. For histological analysis, the entire aortic root area was serially sectioned at 10 μm intervals from the base of the aortic sinus and mounted on slides (SuperFrost Plus, Fisher, Pittsburgh, PA). For each mouse, the average of 3 sections spaced by 50 μm where 3 leaflets are clearly visible was used for quantification. Aortic root lesion area and neutral lipid content were quantified in oil-red o (ORO) stained sections. Lesional collagen content and type were quantified after picro-sirius red staining and visualization under brightfield and polarized light as previously described 6. Collagen types were distinguished by their different colors under polarized light, yellow-red and green for collagen type I and III respectively 7. Smooth muscle cells (SMC) and macrophages within atheroma were identified by immunofluorescence simultaneously using rabbit anti-alpha actin-Cy3 antibody (1/200, Sigma, MO) and rat-anti mouse MOMA-2 (1/200, Cedarlane, Laboratories Limited, ON, CA), followed by incubation with Alexa488 conjugated anti-rat secondary antibodies (1:1000, Invitrogen). TUNEL staining was carried out using the TMR red kit (Roche). Nuclei were stained with Hoescht 33342 (1:10 000; Invitrogen) and slides were mounted in SlowFade Gold (Invitrogen). Sections were viewed with a Zeiss AxioObserver microscope, digitized with a Retiga-SRV CCD camera (Qimaging, Surrey, BC, Canada). Positive areas for macrophages, SMC, ORO, collagen and intimal lesion areas were quantified using Metamorph software (Molecular Devices Inc., Sunnyvale, CA).

**Lipid Extraction and Quantification from Aortic Arch**
At sacrifice, aortic arches were harvested. Adventitial fat was removed and samples were flash-frozen. Lipids were extracted according to the method of Folch et al. 8. Briefly, aorta were minced and mixed overnight with chloroform:methanol (2:1). After addition of 0.9% NaCl, the aqueous phase was collected, and dried under nitrogen. Lipids were resuspended in 1% Triton X-100 PBS and quantified by colorimetric assays (Total and Free Cholesterol E, Wako, VA).

**ApoE Detection in Liver**
At sacrifice, a piece of liver was embedded in tissue freezing medium (OCT) and flash-frozen for histological analysis or directly flash-frozen for molecular analysis. ApoE protein was detected by immunofluorescence in 10μm liver cryosections using anti-mouse apoE antibody 9. Apoe expression was assessed by quantitative real time PCR as described below.

**Analysis of Blood Monocytes**
Monocyte concentrations were assessed by flow cytometry in blood obtained by non-terminal retro-orbital bleeding from 4h-fasted mice. Blood aliquots were mixed with EDTA and kept at room temperature for the entire staining procedure. After a pre-incubation step to block CD16/CD32 (Fc Block, BD Pharmigen), blood cell suspensions were stained with CD45 (30-F11), Ly-6G (1A8), CD11b (M170) and Ly-6C (AL-21) (BD Biosciences). After red blood cell lysis (BD Red Cell Lysing Solution, BD Pharmigen) and fixation, samples were kept on ice and analyzed with a C6 Flow Cytometer (Accuri Cytometers Inc., MI) providing 4 color detection, linear forward scatter and side scatter detections and sample volume determination. Analysis was performed with FlowJo software (Tree Star Inc., OR) using specific gates as indicated in Figure I (available in supplemental material).

**Laser Capture Microdissection of Lesional Macrophages**
Laser capture microdissection was performed under RNase-free conditions as previously described 1. Briefly, the entire aortic root area was serially sectioned at 20 μm intervals from the base of the aortic sinus and mounted on Membrane Slides 1.0 PEN (Carl Zeiss AG, Germany). Every 5th aortic root cross-sections was mounted on SuperFrost Plus slides and stained for MOMA-2 and used as “guide slide” to identify lesional macrophages in surrounding cross-sections microdissected as illustrated in Figure II (available in supplemental material). Sections to be microdissected were fixed in 70% ethanol, counterstained with Hematoxylin QS (Vector Labs), dehydrated in graded ethanol (70%, 95% and 100%), air dried and microdissected with a P.A.L.M. system. Dissected macrophages obtained from each mouse were pooled for RNA isolation (n=6 mice per group).
RNA Extraction and Gene Expression
Total RNA was extracted from microdissected lesional macrophages or from livers using RNeasy Micro or Mini kits and on-column Dnase I treatments (Qiagen, Inc.). For macrophages, RNA concentration was determined by the Quant-iT RiboGreen RNA Kit (Molecular Probes, Carlsbad, CA) and RNA quality was verified with the Agilent 2100 Bioanalyzer and a RNA 6000 Pico Kit (Agilent, CA) (RNA integrity number was 7.2 to 8.8). For liver samples, RNA concentration and quality were assessed using NanoDrop 2000 (Thermo Scientific, DE). Gene mRNA levels were determined using a SYBR OpenArray Cardiovascular Panel (Applied Biosystems) on reverse-transcribed, amplified RNA (Ovation Pico WTA system, Nugen) or using specific primer pairs (Assay-On-Demand, Applied Biosystems) on reverse-transcribed non-amplified RNA (iScript, Bio-Rad Laboratories) (10-25pg for lesional macrophages and 5ng for liver samples per reaction), and an ABI Prism 7900. mRNA levels were normalized to the average housekeeping genes (peptidylprolyl isomerase A (Ppia), beta-glucuronidase (Gusb), beta-2-microglobulin (B2m) and heat shock protein 90-beta (Hsp90ab)) and were calculated according to the 2^ΔΔCt method.

Statistical Analysis
Data are presented as mean±SEM and were analyzed with GraphPad Prism 5 software (GraphPad Software inc., La Jolla, CA). Statistical differences within the 20- or 27-week old cohort were evaluated by one-way ANOVA with Dunnett’s post-test for differences between each experimental group and the control baseline group. In some cases, two-tailed Student t-tests were used as described in figure legends. P values < 0.05 were considered significant.

REFERENCES