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Journal

ISSN

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Publication Date

DOI

Peer reviewed

Inducible *Apoe* Gene Repair in Hypomorphic ApoE Mice Deficient in the LDL Receptor Promotes Atheroma Stabilization with a Human-like Lipoprotein Profile

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Abstract

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

Approach and Results—Chow-fed hypomorphic *Apoe* mice deficient in LDL receptor expression (*Apoe$^{h/h}$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-HDL:HDL-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 extra-cellular matrix remodeling and cell migration were 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 *Apoe$^{h/h}$Ldlr$^{-/-}$Mx1-cre* mice resulted in lesion stabilization in the context of a human-like ratio of non-HDL:HDL-cholesterol. Our data suggest that macrophage loss derived in part from reduced blood Ly-6C^{hi} monocytes levels and genetic reprogramming of lesional macrophages.

Keywords

Apolipoprotein E; Hypomorphic ApoE mice; Atherosclerosis regression; Lesion stabilization; Monocytes; Macrophages

Introduction

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 [2]. 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 [3]. Although lesion remodeling was observed in these clinical trials, plaque shrinkage and prevention of cardiovascular events remained modest [3].

The search for new treatments to promote atherosclerosis regression in humans has led to the development of mouse models in which cellular and molecular mechanisms can be more readily investigated. Plaque regression and stabilization, characterized mainly by a reduction in macrophage content and the accumulation of collagen, has been achieved in mouse models of atherosclerosis in which interventions exerted a drastic and lasting reduction of diet-induced hypercholesterolemia [4]. Because mice respond poorly to the cholesterol-lowering effect of statins [4], multiple strategies, ranging from aortic arch transplant [5] to transgene induction [6-11] 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 CCR7 (C-C chemokine receptor type 7)-dependent mechanism [10, 12, 14]. However, in another study using a different mouse model[15], reduced macrophage content during plaque 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 (HDL) and very low levels of plasma apoB-lipoproteins (or non-HDL lipoproteins) [4]. 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 ($Apoe^{h/h}Mx1-cre$) also refered to as HypoE mice [16]. The hypomorphic $Apoe$ allele results in low $Apoe$ expression levels (5-10% of WT levels) [16]. HypoE mice fed a chow diet display a lipoprotein profile dominated by HDL 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 [8, 16]. More recently, we have reported that HypoE mice deficient in LDL receptor ($Apoeh/hLdlr^{-/-}Mx1-cre$ mice) develop spontaneous hyperlipidemia and atherosclerosis on a chow diet [17].

Here, we show that the inducible cre-mediated repair of the hypomorphic $Apoe$ allele in $Apoe^{h/h}Ldlr^{-/-} Mx1-cre$ mice restored liver apoE expression and caused a reduction in plasma cholesterol and triglyceride levels, characterized by moderately elevated levels of apoB-lipoprotein and a human-like ratio of non-HDL:HDL-cholesterol [18]. These plasma lipid changes occurred in mice which were fed a chow diet throughout the entire study, allowing us to study atheroma remodeling in the absence of potential confounding effects due to feeding or withdrawal of a high fat diet.

Materials and Methods

Available online at http://atvb.ahajournals.org.

Results

Inducible repair of the hypomorphic *Apoe* allele in *Apoe^{h/h}Ldlr^{−/−}Mx1-cre* mice restores hepatic apoE expression, reduces hyperlipidemia and improves the ratio of non-HDL:HDL-cholesterol

By twenty weeks of age, chow-fed *Apoe^{h/h}Ldlr^{−/−}Mx1-cre* mice displayed severe hypercholesterolemia and hypertriglyceridemia (719 ± 28 mg/dl 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 two intra-peritoneal injections of polyinosinic-polycytidylic ribonucleic acid (pIpC) one day apart. One week after pIpC treatment, *Apoe* mRNA expression levels increased by 5-fold in the liver whereas *Apoe* mRNA levels were not changed after saline injections (Figure 2B). While apoE immunoreactivity was mainly detected in hepatic sinusoids at baseline, hepatocytes displayed increased intracellular staining after pIpC injections (Figure 2A), indicating that pIpC treatment effectively repaired the hypomorphic *Apoe* allele in *Apoe^{h/h} Ldlr^{−/−}Mx1-cre* mice.

One week after pIpC-treatment, both total plasma cholesterol and triglyceride levels were decreased by 2.1-fold to 337 ± 27 mg/dl and 182 ± 15 mg/dl respectively (Figure 1A and 1B). Plasma lipid lowering persisted to the 4-week time point in pIpC-treated mice while no change was observed in control saline-treated mice (Figure 1A and B). 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, pIpC-treated mice displayed a 3.8-fold decrease in apoB-lipoprotein-cholesterol levels (Table I). Specifically, levels of VLDL- and IDL/LDL-cholesterol were reduced by 5.7- and 2.6-fold respectively (Figure 1D and Table I). In addition, plasma apoB-48 and apoB-100 levels were reduced by 3.7- and 1.2-fold respectively (Figure 1C). Concomitantly, pIpC-induced mice showed an increase in plasma HDL-cholesterol and apoA1 levels by 1.4- and 1.6-fold respectively (Figure 1D, 1C and Table I). While we observed a change in the distribution of apoE among plasma lipoproteins before and after intervention, total plasma apoE levels in plasma remained unchanged (Figure 2C and 2D, Figure 1C). The repair of the hypomorphic *Apoe* allele was characterized by an enrichment of apoE in large HDL that eluted in fractions 18 to 20 following FPLC (Figure 2C) and were found within the 1.02-1.06 g/ml lipoprotein density fraction following density ultracentrifugation (Figure 2D). Overall, within one week of pIpC-treatment the drop in cholesterol among apoB-lipoproteins and rise among HDL led to a decrease of the non-HDL: HDL-cholesterol ratio from 7 (87%:13%) to 1.5 (60%:40%) (Table I and Figure 1). These changes in plasma lipids and lipoprotein levels persisted 4 weeks after pIpC-treatment, were independent of any change in body weight or blood glucose level (Figure III) and occurred in mice continously fed a chow diet.

Apoe gene repair halts atherosclerosis progression in *Apoe^{h/h}Ldlr^{−/−}Mx1-cre* mice

Twenty-week old mice displayed aortic root atherosclerosis that was mainly composed of multi-layered foam cells along with occasional collagen fibrous caps. By 27 weeks of age, atherosclerotic lesions had doubled in size (Figure 3A and 3C) and atheromas were more advanced, containing thicker layers of macrophages with an increased proportion of smooth muscle cells and fibrous caps (Figure 3C and 4C). Because lesion stage can influence the susceptibility of atheroma remodeling, we examined changes in both 20- and 27-week old baseline mice, bearing features of early and advanced lesions respectively. Aortic root lesion sizes were determined in separate groups of mice sacrificed either at the baseline time points (20- or 27-week old) or several weeks after pIpC-injections. Measurements of lesion 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 to 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 to 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 up to 8 weeks after intervention. Lesion sizes were not different from baseline (Figure IV). 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, 3C and IVB). High power magnification of oil-red O stained sections revealed neutral lipid deposits that appeared to localized both within and outside of residual foam cells (Figure V). 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.

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. While the content of cholesterol esters (CE) and free cholesterol (FC) tended to increase in aortic arches of saline-treated mice (p=0.09), there was no change in pIpC-treated mice compared to baseline levels (Figure 3D). Overall these results indicate that repairing the hypomorphic *Apoe* allele in *Apoe^{h/h}Ldlr^{-/-}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^{h/h}Ldlr^{-/-}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 pIpC injection compared to baseline (−18% and −37% for the 20- and 27-week old groups respectively), while 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 up to 8 weeks following *Apoe* gene repair in 20-week old mice and in fact was accentuated at this timepoint (−32% and +135% respectively, Figure IVC, D and F, Table IV). 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 pIpC- and saline-treated mice clearly differed in their relative proportion of intimal collagen and macrophages with a higher ratio of collagen to macrophages in pIpC-treated mice compared to 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 pIpC- and saline-treated mice (Figure 4C and VI). Lesions of pIpC-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 [19]. In contrast, lesions of saline control mice contained more diffuse networks of green type III collagen fibers surrounding foam cells, known for their extensibility [19]. The proportion of type I to type III collagen was quantitavely increased in lesions of pIpC-treated mice compared to those of saline control mice in the 27-week old group (Figure VIB). No difference was observed in the 20-week old group (Figure VIA), suggesting a lesion stage specific effect. Overall, these quantitative and qualitative analyses indicate that *Apoe* gene repair in *Apoe^{h/h}Ldlr^{-/-}Mx1-cre* mice was associated with increased markers of lesion stability.

Apoe gene repair does not impact lesional macrophage apoptosis in *Apoe^{h/h}Ldlr^{−/−}Mx1-cre* mice

To address wether 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 pIpC 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 mm^2 of MOMA-2 area) was unchanged between baseline and pIpC-injected mice in both 20- and 27-week old cohorts.

ApoE-dependent plasma lipid lowering in *Apoe^{h/h}Ldlr^{−/−}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 [10, 12, 13]. 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 to those isolated 1 week after treatment. No significant changes were observed in mRNA levels of genes related to cholesterol efflux (*Lxr*, *Lxr*, *Abca1*, *Abcg1*) (Figure 5D), M1 inflammatory (*Tnf*, *Mcp1*) or M2 anti-inflammatory responses (*Mgl1*, *Tgf*) (Figure 5E). In addition, the expression of *Ccr7*, previously implicated in macrophage egress during plaque remodeling [13], 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 PCR 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 and Table II, available online). Among these, several genes were associated with extra-cellular 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 beta 2 (*Itgb2* by 1.27-fold) (Figure 5F and Table II). Of note, mRNA expression levels of pro-apoptotic (*Bax*, *Caspase-3* and *6*) and anti-apoptotic (*Bcl2* and *Bcl-xL*) genes, present on the OpenArray, were unchanged 1 week after lipid lowering (Table III).

Apoe gene repair decreases blood Ly-6C^{high} monocytes in *Apoe^{h/h}Ldlr^{−/−}Mx1-cre* mice

Because blood monocyte recruitment into plaques is strongly affected by monocyte levels in the circulation [20], we examined whether restoration of apoE expression affected blood monocyte levels in the 27-week-old group of mice. As shown in Figure 6A, a 30% decrease in the number of total circulating monocytes was observed two weeks after pIpC-induced *Apoe* re-expression, while there was no reduction in saline-treated mice. The reduction in blood monocytes derived mainly from a 64% decrease in the number of Ly-6C^{high} monocyte subsets (Figure 6B) while the number of Ly-6C^{low} monocytes did not change significantly (Figure 6C). There was no detectable change in the numbers of Ly-6C^{high} 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*$^{h/h}$*Ldlr*$^{-/-}$*Mx1-cre* mice develop spontaneous hyperlipidemia and atherosclerosis on a chow diet [17]. Here, we show that the inducible repair of the hypomorphic *Apoe* alleles in these mice caused: 1) plasma lipid reduction characterized by a decrease of the non-HDL:HDL-cholesterol ratio from 7 to 1.5; 2) arrest of atheroclerosis progression; 3) progressive loss of lesional macrophages and accumulation of thick collagen fibers, hallmarks of plaque stabilization; 4) unchanged frequency of apoptotic lesional macrophages; 5) changes in the expression of genes involved in extra-cellular matrix remodeling and cell migration in lesional macrophages ; 6) no significant changes in the expression of *Ccr7* and genes involved in cholesterol efflux and inflammation in lesional macrophages one week after plasma lipid lowering; and 7) decreased numbers of circulating Ly-6C^{hi} monocytes.

First, we observed that restoring normal apoE expression in chow-fed hypomorphic *Apoe*$^{h/h}$*Ldlr*$^{-/-}$*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 [21]. Inducible *Apoe* gene repair in these mice also led to a rise in plasma HDL-cholesterol and apoAI levels accompanied by enrichment in large apoE-rich HDL supporting the proposed role of apoE in promoting HDL expansion and remodeling [22]. 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 [4] as they remained within the range of a lipoprotein ratio seen in humans [18].

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 [3] and mice [5, 6, 9, 11] including our prior studies of HypoE mice [8] 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 (≈200mg/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 [10] or in *Ldlr*$^{-/-}$ mice switched from a HFD to a chow diet [9, 23]. Conversely, lesion regression was reported in mice despite very high level of non-HDL-cholesterol levels [7, 24]. Thus, taken together, our study and those of others suggest that lesion regression is influenced by a number of 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 apoAI overexpression [11, 25, 26]. 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 aortic arch were 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 (*Lxr* , *Lxr* , *Abca1*, *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.* [11] recently reported plaque remodeling with a reduction in macrophage content without net neutral lipid loss in *Ldlr*$^{-/-}$ mice following LDL-receptor transgene induction. This is in

contrast with other studies reporting that macrophage loss occurred concurrently [5, 6, 10] or preceded [15] arterial neutral lipid loss, and was associated with increased expression of genes involved in cholesterol efflux [12]. In the latter studies, hyperlipidemia lowering was triggered by diet change [10, 12] or by virus-derived apoE expression in $Apoe^{-/-}$ mice [6, 15]. Thus, it is possible that the withdrawal of diets rich in fat or an over-expression of apoE itself rapidly induced the removal of arterial lipids independently of an effect on lesional macrophage loss.

Levels of monocytes in the circulation strongly affect the rate of their infiltration in the vessel wall where they transform into plaque macrophages [20]. We found that the Ly-6C^{high} monocyte subset was reduced after $Apoe$ $gene$ repair. Ly-6C^{high} monocytes are known to preferentially accumulate in growing atheroma in hyperlipidemic mice [27]. The frequency of apoptotic lesional macrophages was unchanged after apoE restoration. So, it is possible that decreased Ly-6C^{high} monocyte recruitment, coupled with a stable rate of macrophage apoptosis, led to reduced lesional macrophage content in our model. Interestingly, Murphy et al [28] recently demonstrated that cell-derived apoE inhibits hematopoietic stem cell proliferation and monocytosis in hyperlipidemic mice. Thus, the inducible cre-mediated repair of the hypomorphic $Apoe$ allele in $Apoe^{h/h}Ldlr^{-/-}Mx1$-cre mice may have affected hematopoietic stem cell proliferation and monocytosis. The potential importance of blood monocytes in modulating lesional macrophage loss during plaque remodeling was highlighted in two recent studies [15, 23]. First, a decrease in blood monocyte levels was observed in $Ldlr^{-/-}$ mice fed an atherogenic diet followed by a switch to a low fat diet [23]. Second, Potteaux et $al.$ [15] recently demonstrated that suppressed monocyte activation and recruitement coupled with a stable rate of apoptosis accounted for macrophage loss in regressing lesions of HFD-fed $Apoe^{-/-}$ mice treated with apoE-encoding adenoviral vectors. Monocytosis is an independent risk factor for atherosclerosis progression in humans [29]. 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 [12, 14] and the Reversa mouse [10] showed that macrophage emigration to regional and systemic lymph nodes participated in their removal during lesion regression and remodeling. This process relied on LXR-dependent upregulation of the chemokine receptor CCR7 in macrophages which confers dentritic cell-like features [30]. Van Craeyveld et al. [11] recently failed to detect increased $Ccr7$ expression during plaque remodeling in $Ldlr^{-/-}$ mice 12 weeks following 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 Lxr 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, 3 or 14 days after lipoprotein profile correction respectively [10, 12]. Other factors could also account for the differences between our results and earlier studies [10, 12]. 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 $al.$ [15] recently demonstrated that lesional macrophage loss during lesion remodeling was independent of a role of CCR7-induced egress in the HFD-fed $Apoe^{-/-/-}$ mouse after apoE was reintroduced by liver-directed gene transfer. Collectively, these observations suggest that plaque macrophage loss could occur independently from the up-regulation 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 up-regulation of matrix metalloproteinases such as *Mmp2*, involved in the breakdown of collagen [31]. Interestingly, in our model, the expression of *Mmp2* was decreased in lesional macrophages one week after *Apoe* gene repair, supporting the concept that lesional macrophages undergo an active genetic reprogramming that likely contribute to collagen accumulation and lesion stabilization. Increases in the expression of genes involved in cell mobility such as *Mmp19* [32] or *Vasp* [33] or cell adhesion such as *Vcan* [34] 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^{h/h}Ldlr^{−/−}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^{hi} monocyte levels and genetic reprogramming of lesional macrophages.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Significance

We introduce HypoE mice deficient in LDL receptor expression ($Apoe^{h/h}LDLr^{-/-}$ 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 LDL while increasing apoA1 and apoE in HDL. These changes suppress the expansion of activated blood monocytes and promote atheroma stabilization within one 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 suggest 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.
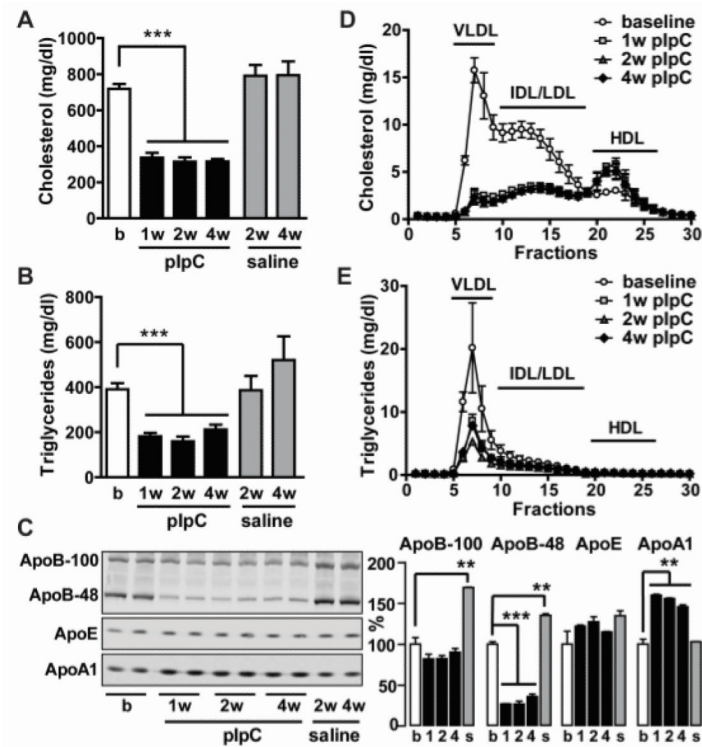
Figure 1.
Plasma cholesterol (**A**) and triglyceride (**B**) levels in mice at baseline and after pIpC or
saline injections (n=7-23 per group). Plasma apolipoprotein levels (**C**) (Western blot and
quantification, n=2 pools of 4 mice each). Cholesterol (**D**) and triglyceride (**E**) distribution
in plasma lipoprotein separated by FPLC (n=2 pools of 4 mice each). Mean±SEM.
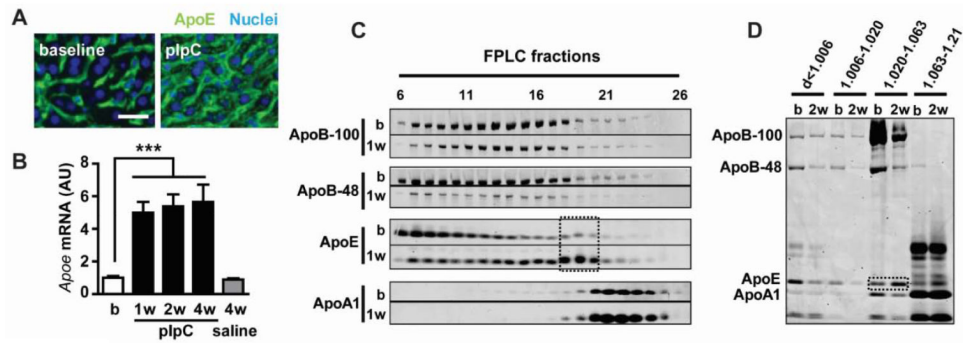**p<0.01, ***p<0.001 versus baseline by Dunnett's test post-ANOVA; b is for baseline; s is
for saline

Figure 2.
ApoE protein (**A**) (Representative images, Scale bar=50μm) and apoE mRNA levels (**B**) in mouse livers (n=4 per group; AU, Arbitrary Unit; b is for baseline). Mean±SEM. ***p<0.001 versus baseline. Apolipoprotein distribution in plasma lipoprotein separated by FPLC (**C**) or ultracentrifugation (**D**) with large HDL ApoE-enriched particules (dashlined box)
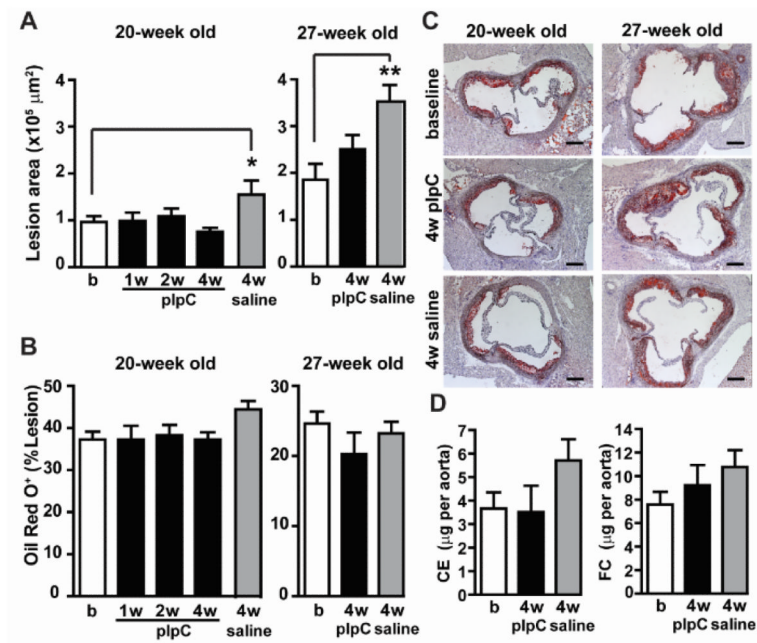
Figure 3.
Area (**A**) and neutral lipid content (**B**) of aortic root lesions from 20- and 27-week old group (n=6-16 for each time point). Representative Oil Red O-stained sections (**C**) (Scale bar=200μm). Aortic arch lipid levels (**D**) (n=8 per group, cholesterol esters (CE), free cholesterol (FC)). Mean±SEM, *p<0.05, **p<0.01 versus baseline by Dunnett's test post-ANOVA
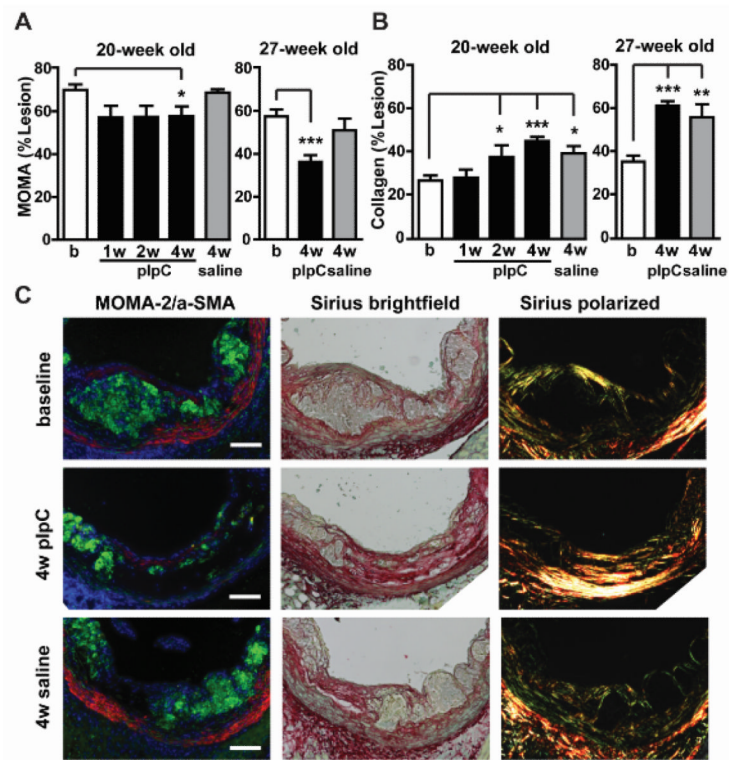
Figure 4.
Macrophage (**A**) and collagen (**B**) content of aortic root lesions from 20- and 27-week old group (n=6-16 for each time point). Mean±SEM, *p<0.05, **p<0.01, ***p<0.001 versus baseline by Dunnett's test post-ANOVA. Representative cross-sections (**C**) with MOMA-2^{+} macrophages (green), smooth muscle cells (red), nuclei (blue), and sirius-red-stained collagen viewed under bright or polarized light (Scale bar=100μm)
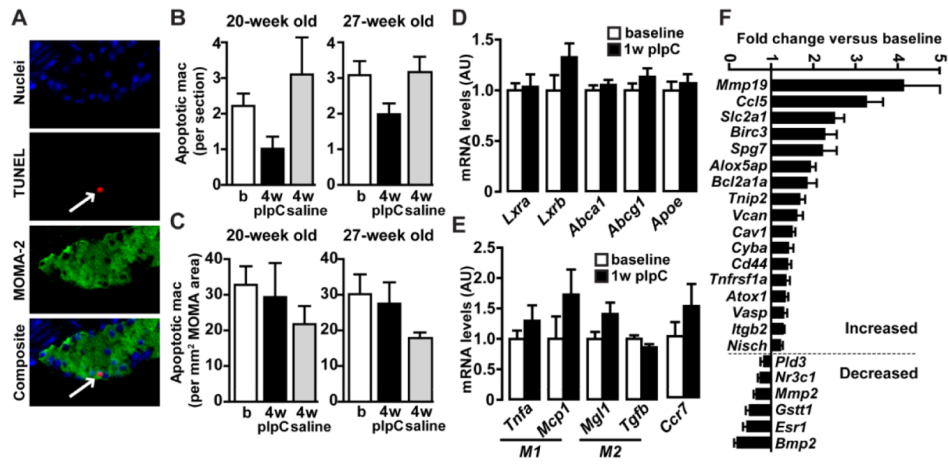
Figure 5.
(**A**) Apoptotic macrophages, detected as hoescht+ (blue), TUNEL+ (red) and MOMA-2+ (green) were counted (**B**) per section and (**C**) normalized per mm2 MOMA area (n=5-6 per group, Mean±SEM). Lesional macrophage mRNA levels of genes related to (**D**) cholesterol efflux, (**E**) M1/M2 responses and egress (Relative to baseline, AU: Arbitrary Unit, n=8 per group, Mean±SEM). (**F**) Genes from OpenArray Cardiovascular panel significantly increased or decreased 1 week after pIpC-treatment (Fold change versus baseline, n=6 per group, Mean±SD, p<0.05 by t-test for all).
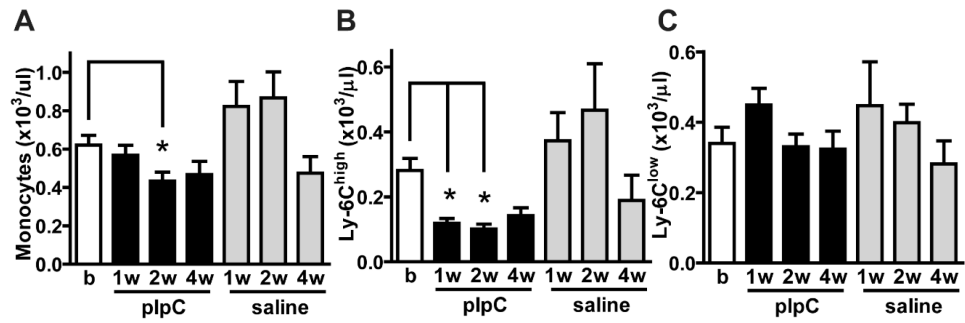
Figure 6.
Counts of total blood monocytes (**A**), Ly-6C^{high} (**B**) and Ly-6C^{low} (**C**) subsets in 27-week old mice at baseline and after pIpC or saline treatment (n=7-20 per group). Mean±SEM. *p<0.05 versus baseline by t-test (A) or by Dunnett's test post-ANOVA (B).