Chapter 7

Bone marrow reconstitution in ApoE−/− mice: a novel model to induce atherosclerotic plaque regression

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ABSTRACT

Background & Aims: While numerous studies have been dedicated to inhibit the development and progression of atherosclerosis, recent attention has been drawn to the goal of reversing atherosclerosis, meaning regressing of pre-existing atherosclerotic plaques. The aim of this study is to investigate the potential of combined macrophage-specific apoE production and LXR agonist treatment to induce atherosclerotic plaque regression.

Methods and Results: ApoE<sup>−/−</sup> mice were fed with regular chow diet for 16 weeks and then switched to an atherogenic diet for another 3 days or 3 weeks to develop initial or advanced atherosclerotic lesions. We used bone marrow transplantation technique, reconstituting ApoE<sup>−/−</sup> mice with bone marrow from C57BL/6 mice, to restore apoE function in macrophages and normalize plasma lipoprotein profiles. Combined with LXR agonist T0901317, we evaluated the potential of LXR activation to regress diet-induced pre-existing atherosclerotic plaques.

Conclusions: Our study shows that 1) ApoE<sup>−/−</sup> mice reconstituted with bone marrow from C57BL/6 mice represents a promising mouse model with chow diet feeding to study atherosclerosis regression, providing an alternative model to investigate plaque regression; and 2) rapidly optimized plasma lipoprotein profiles, combined with LXR agonist treatment, induced favorable gene expression profiles that can induce significant regression of both initial and more advanced atherosclerotic plaques.

Keywords: LXR, T0901317, bone marrow transplantation, apoE, lipoprotein, atherosclerosis, regression
INTRODUCTION

Hypercholesterolemia plays a key role in the development of atherosclerosis and is a causative factor for coronary artery disease. Hyperlipidemia is a metabolic disorder defined by elevated levels of plasma low-density lipoprotein (LDL)-cholesterol and triglycerides concentrations, and/or decreased levels of the atheroprotective high-density lipoprotein (HDL)-cholesterol and its major protein component apolipoprotein Al (apoA-I). Lowering of very-low-density lipoprotein (VLDL)- and low-density lipoprotein (LDL)-cholesterol levels leads to a reduction in cardiovascular morbidity and mortality. In contrast, high levels of HDL-cholesterol are associated with a decreased risk of cardiovascular disease. HDL serves anti-atherogenic functions because of its ability to mediate reverse cholesterol transport (RCT). RCT involves the HDL mediated removal of cholesterol from the periphery, allowing it to be cleared by the liver and then excreted into bile. Modulation of major macrophage mediators in RCT, such as ATP-binding cassette transporter A1 (ABCA1), ATP-binding cassette transporter G1 (ABCG1), and scavenger receptor class BI (SR-BI) has been considered as promising strategies for the prevention of atherosclerosis.

Hematopoietic cells, in particular monocytes and macrophages, play integral roles at all stages of atherosclerosis. The lipid-laden macrophage-derived foam cells are present from the earliest discernable fatty-streak lesions to advanced plaques, and are key factors in the pathology of plaques. Much of the work exploring the role of macrophages in atherosclerosis has been carried out using the murine bone marrow transplantation model in which recipient mice prone to atherosclerosis development are reconstituted with donor bone marrow cells from transgenic or knockout mice to over express or delete genes in macrophages in a relevant pathway.

ApoE-deficient (ApoE<sup>−/−</sup>) mice are one of the most common animal models to study atherogenesis. ApoE<sup>−/−</sup> mice show impaired clearance of plasma lipoproteins. The most obvious phenotype of ApoE<sup>−/−</sup> mice is the spontaneous development of atherosclerotic lesions, even on a regular chow diet which is low in fat content and does not contain added cholesterol. Lesions of ApoE<sup>−/−</sup> mice develop over time from initial fatty streaks to complex lesions, and this process can be strongly accelerated by a high-fat, high-cholesterol diet. ApoE is a major component of several classes of plasma lipoproteins. Increasing evidence from both animal and human studies suggests that apoE is able to protect not only against hyperlipidemia, but also against atherosclerosis via a variety of mechanisms, including promoting efficient uptake of triglyceride-rich lipoproteins from the circulation by peripheral tissues for utilization or by liver for excretion, maintaining normal macrophage lipid homeostasis, and enhancing RCT from macrophage foam cells in the atherosclerotic lesion. Although the majority of apoE in plasma originates from the liver, apoE is synthesized by a variety of peripheral tissues and cell types, including macrophages. Previous studies have shown that reconstruction of macrophage-specific expression of apoE reduces atherosclerosis in ApoE<sup>−/−</sup> mice, whereas reconstitution of C57BL/6 mice with macrophages from ApoE<sup>−/−</sup> mice increases atherosclerosis, suggesting that apoE produced by macrophages was sufficient to induce changes in atherosclerotic development.

While numerous studies have been dedicated to inhibit the development and
progression of atherosclerosis, recent attention has been drawn to the goal of reversing atherosclerosis, meaning regressing of pre-existing atherosclerotic plaques. The first evidence of dramatic atherosclerotic regression in mice was achieved via robust surgical measures to rapidly improve the plaque environment. This study suggested that the essential prerequisite for promoting regression of atherosclerotic lesions is robust improvement of plasma lipoprotein profiles and plaque milieu, including large plasma reductions in atherogenic apoB-lipoproteins and brisk enhancements in efflux of cholesterol from plaques to the liver. Recently, Feig et al showed that the LXR agonist T0901317 promotes egress of monocyte-derived cells from mouse aortic plaques, indicating that LXR is required for maximal effects on plaque macrophage egress during atherosclerosis regression in mice. Liver X receptors (LXRs) are sterol-responsive transcription factors which regulate expression of genes involved in cholesterol metabolism and homeostasis. LXRs act as cholesterol sensors. When cellular oxysterols accumulate as a result of increasing concentrations of cholesterol, LXR induces the transcription of genes that protect cells from cholesterol overload. LXR activation has been shown to significantly promote biliary sterol secretion and reduce cholesterol absorption, up-regulate cholesterol efflux to HDL particles, and inhibit development of atherosclerosis, providing direct evidence for an anti-atherogenic effect of LXR agonists. However, in those studies, LXR agonists are only shown to attenuate the progression of atherosclerosis in mouse models, while their potential to abrogate pre-existing cardiovascular disease and to stabilize established atherosclerotic lesions has not been widely addressed. It is thus clinically interesting to examine whether rapidly improved plasma lipoprotein profiles combined with therapeutic LXR agonist could induce atherosclerotic lesion regression.

In the current study, we used bone marrow transplantation technique, reconstituting ApoE−/− mice with bone marrow from C57BL/6 mice, to restore apoE function in macrophages and normalize plasma lipoprotein profiles. Combined with LXR agonist T0901317, we evaluated the potential of LXR activation to regress diet-induced pre-existing atherosclerotic plaques.

MATERIALS AND METHODS

Animals
Female homozygous ApoE-deficient (ApoE−/−; C57BL/6 background) mice of 12 weeks old were used. To study the effects on initial atherosclerotic plaques, mice were fed with semi-synthetic Western-type diet (WTD) containing 15% (w/w) fat and 0.25% (w/w) cholesterol (Diet W, Special Diet Services, Witham, UK) for 3 days to induce the development of initial atherosclerotic lesions. To study the effects on advanced atherosclerotic plaques, the animals were fed with WTD for 3 weeks to induce the further development of advanced atherosclerotic lesions. After the formation of atherosclerotic plaques, in both studies, bone marrow transplantation was performed and the diet was switched to regular cholesterol-free chow diet containing 4.3% (w/w) fat (RM3, Special Diet Services, Witham, UK) for 6 weeks, with or without supplementation of the LXR agonist T0901317 (10 mg/kg/day; MSD Oss, The Netherlands). After euthanization, mice were bled via orbital exsanguination, and perfused in situ through the left cardiac ventricle with
ice-cold PBS (pH 7.4) for 20 minutes. Tissues were dissected and snap-frozen in
liquid nitrogen. Heart was dissected free of fat and stored in 3.7% neutral-buffered
formalin (Formal-fixx, Shandon Scientific Ltd., UK) for histological analysis. Animal
care and procedures were performed in accordance with the national guidelines for
animal experimentation. All protocols were approved by the Ethics Committee for
Animal Experiments of Leiden University.

Bone marrow transplantation
From one week before bone marrow transplantation, recipient female ApoE<sup>–/–</sup>
mice were kept on antibiotics-containing drinking water (83 mg/L ciprofloxacin, 67 mg/L
polymyxin B sulfate, 6.5 g/L sucrose). To induce bone marrow aplasia, recipient
ApoE<sup>–/–</sup> mice were exposed to a single dose of 9 Gy (0.19 Gy/min, 200 kV, 4 mA)
total body X-ray irradiation, using an Andrex Smart 225 Röntgen source (YXLON
International, Copenhagen, Denmark) with a 6-mm aluminum filter. Bone marrow
from donor female C57BL/6 mice was harvested by flushing the femurs and tibias
with PBS (pH 7.4). Single-cell suspensions were prepared by passing the cells
through a 70 µm cell strainer (BD, Breda, The Netherlands). 0.5 x 10<sup>7</sup> donor bone
marrow cells were injected intravenously into the lateral tail vein of each irradiated
recipient mouse. All transplanted mice were housed in sterilized filter-top cages
with drinking water containing antibiotics throughout the whole experiment. After
the mice were euthanized at 6 weeks of regular chow diet feeding, the
hematological chimerism of the transplanted mouse was assessed by polymerase
chain reaction (PCR) analysis of DNA harvested from bone marrow to detect the
presence of the apoE allele.

Plasma lipid analysis
Plasma lipid analysis was performed at different time points throughout the
experiments. At the endpoint, mice were not fasted prior to euthanization. Plasma
concentrations of total cholesterol (TC) and triglycerides (TG) were measured
using the enzymatic colorimetric assay (Roche Diagnostics, Mannheim, Germany).
The distribution of cholesterol over different lipoproteins in plasma was determined
by fast protein liquid chromatography (FPLC) through a Superose 6 column (3.2 x
30 mm; Smart-System, Pharmacia, Uppsala, Sweden). Cholesterol content of the
lipoprotein fractions was determined as described above.

RNA isolation and gene expression analysis
Total RNA from the liver was isolated using acid guanidinium thiocyanate (GTC)-
phenol-chloroform extraction. Briefly, 500 µL of GTC solution (4 M guanidine
isothiocyanate, 25 mM sodium citrate, 0.5% N-lauroylsarcosine) was added to
each sample, followed by acid phenol:chloroform extraction. The RNA in the
aqueous phase was precipitated with isopropanol. The quantity and purity of the
isolated RNA were examined using an ND-1000 Spectrophotometer (Nanodrop,
Wilmington, DE, USA). One microgram of the isolated RNA from each sample was
converted into cDNA by reverse transcription with RevertAid™ M-MuLV Reverse
Transcriptase (Promega, Madison, WI, USA). Negative controls without addition of
reverse transcriptase were prepared for each sample. Quantitative real-time PCR
was carried out using ABI Prism 7700 Sequence Detection system (Applied
Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.
36B4, Beta-actin, and GAPDH were used as internal housekeeping genes. The
gene-specific primer sequences used are listed in Table 1. Amplification curves were analyzed using 7500 Fast System SDS software V1.4 (Applied Biosystems, Foster City, CA, USA). The relative expression of each gene was expressed as fold changes compared to baseline group.

<table>
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<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<td>Cd6b</td>
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<td>TTGGGTATGAGATCCGAGTATGG</td>
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Histological analysis
Accumulation of lipids in the atherosclerotic plaques at the aortic root in the heart was analyzed. The heart was cut latitudinally and embedded in O.C.T™ Compound (Tissue-Tek, Sakura finetek, Tokyo, Japan), and subsequently sectioned using a Leica CM 3050S cryostat at 10 µm intervals. Cryostat sections were stained with Oil-red O (Sigma-Aldrich) to identify lipids, and counterstained with hematoxylin (Sigma-Aldrich) to assist in tissue visualization. Quantitative analysis of the atherosclerotic lesion area at the aortic root was performed in a blinded fashion. Mean lesion area (in µm² per aortic root per mouse) was calculated from 10 Oil-red O stained cryostat sections, starting at the appearance of the tricuspid valves.

Immunohistochemistry
Ten-micrometer cryosections of the aortic root were obtained as described above. After incubation with blocking solution (5% goat serum), macrophages were detected using MOMA-2 antibody (rat antibody directed against mouse monocytes/macrophages, Serotec, Oxford, UK). A rabbit anti rat IgG/HRP was used as second antibody (Dako, Heverlee, Belgium). Sections were developed using NovaRED Peroxidase Substrate Kit (VECTOR LABORATORIES, Peterborough, UK) according to kit instructions. Slides were counterstained with hematoxylin (Sigma-Aldrich) to assist in tissue visualization.

Masson’s Trichrome Staining
Tissue sections were prepared as described above and subjected to Masson’s trichrome staining using the Masson’s Trichrome Stain kit (Sigma-Aldrich) and counterstained with hematoxylin, to assess the extent of collagen deposition and the structural integrity of fibrillar collagen in the plaque.

Statistical analysis
Mean values between two groups were analyzed with the unpaired Student’s t-test;
data sets containing multiple groups were analyzed by ANOVA (Instat GraphPad software, San Diego, USA). Statistical significance was defined as p<0.05. Data are expressed as means ± SEM.

RESULTS

To investigate the potential of combined macrophage-specific apoE production and LXR agonist treatment to induce atherosclerotic plaque regression, bone marrow transplantation was performed to selectively express apoE in bone marrow-derived hematopoietic cells, including macrophages. We started our investigation with initial atherosclerotic lesions, which was thought to form an easier target for regression. Here to we fed female ApoE−/− mice with regular chow diet for 16 weeks and then switched to an atherogenic WTD for another 3 days to develop initial atherosclerotic lesions. In addition, we also evaluated the regression of advanced atherosclerotic lesions in the same model. Here we fed female ApoE−/− mice with regular chow diet for 16 weeks and then switched to an atherogenic WTD for another 3 weeks to develop more advanced atherosclerotic lesions. A group of mice were sacrificed to obtain baseline data, whilst the remainder of the mice received bone marrow from either C57BL/6 mice, or from original ApoE−/− mice as a control group for the BMT procedure. After bone marrow transplantation, mice were fed low-fat cholesterol-free chow diet with or without LXR agonist supplementation for 6 weeks, during which the plasma lipid concentrations were monitored over regression period.

Figure 1. Overview of changes in plasma total cholesterol level during regression studies in initial lesions (A) and advanced lesions (B). We fed female ApoE−/− mice with regular chow diet for 16 weeks and then switched to an atherogenic WTD for 3 days (A) or 3 weeks (B) to develop initial and advanced atherosclerotic lesions, respectively. A group of mice were then sacrificed to obtain baseline data, whilst the remainder of the mice received bone marrow from either C57BL/6 mice, or from original ApoE−/− mice as a control group for the BMT procedure. After bone marrow transplantation, mice were fed low-fat cholesterol-free chow diet with or without LXR agonist T0901317 supplementation for 6 weeks, during which the plasma lipid concentrations were monitored. Values are means ± SEM (10 mice per group). BL/6: Bone marrow from wildtype C57BL/6 mice. ApoE−/−: Bone marrow from ApoE−/− mice. T09: LXR agonist T0901317 supplementation.
**Reconstitution of macrophage apoE dramatically normalized plasma lipoprotein profile in ApoE<sup>−/−</sup> mice**

In ApoE<sup>−/−</sup> mice, WTD markedly increased plasma total cholesterol level to approximately 730 mg/dL in the initial lesion study (Figure 1A) and 950 mg/dL in advanced lesion study (Figure 1B). Bone marrow from apoE containing donor C57BL/6 mice was transplanted into ApoE<sup>−/−</sup> mice and a diet switch to low-fat chow diet led to a sharp drop in plasma total cholesterol level within 3 weeks to 188 mg/dL (-74%) in the initial lesion study and 120 mg/dL (-87%) in advanced lesion study, and these levels remained low until 6 weeks after BMT (Figure 1A, 1B). The presence of LXR agonist in chow diet did not change the cholesterol levels, and actually a similar persistent reduction in plasma total cholesterol concentration was noticed (Figure 1A, 1B). In contrast, the cholesterol levels in the control group where ApoE<sup>−/−</sup> mice received bone marrow from original ApoE<sup>−/−</sup> mice started to rise again after an initial drop and displayed a significantly higher plasma total cholesterol level compared to ApoE<sup>−/−</sup> mice with bone marrow from C57BL/6 mice (Figure 1B). At 6 weeks after BMT, in both initial and advanced lesion studies, ApoE<sup>−/−</sup> mice with bone marrow from C57BL/6 mice showed approximately 90% reduction in plasma cholesterol level compared to baseline and there was no significant difference between mice fed chow diet alone or with LXR agonist treatment; Control ApoE<sup>−/−</sup> mice with bone marrow from ApoE<sup>−/−</sup> mice showed significantly higher plasma cholesterol level compared to mice with wildtype C57BL/6 bone marrow, especially when treated with LXR agonist (Figure 2A, 2C). As expected, LXR agonist significantly increased plasma triglycerides levels as compared to mice fed with chow diet alone in both the initial lesion study (1.4-fold, Figure 2B) and advanced lesion study (2.3-fold, Figure 2D).

As determined by FPLC lipoprotein separation, the largely reduced plasma total cholesterol concentration in ApoE<sup>−/−</sup> mice with C57BL/6 bone marrow was primarily due to markedly reduced plasma VLDL-(-94%, \( P < 0.001 \) in initial lesions; -97%, \( P < 0.001 \) in advanced lesions) and LDL- (-82%, \( P < 0.001 \) in initial lesions; -91%, \( P < 0.001 \) in advanced lesions) cholesterol level (Figure 3A, 3C). LXR agonist treatment increased the plasma HDL-cholesterol concentration in ApoE<sup>−/−</sup> mice with C57BL/6 bone marrow compared to baseline (1.6-fold in initial lesions; 1.7-fold, \( P < 0.05 \) in advanced lesions; Figure 3B, 3D). In conclusion, after transplanting bone marrow from C57BL/6 mice into ApoE<sup>−/−</sup> mice, combined with a switch to chow diet with or without LXR agonist, successfully normalized the plasma cholesterol profile.
Figure 2. Plasma concentration of total cholesterol and triglycerides from initial lesion study (A, B) and advanced lesion study (C, D) at endpoint of experiments were measured. Values are means ± SEM (10 mice per group). *P<0.05; ***P<0.001; ns, not significant.

Figure 3. Plasma lipoproteins profile in initial lesion study (A, B) and advanced lesion study (C, D) at endpoint of experiments. Plasma lipoproteins were separated by FPLC and cholesterol level was measured in each fraction. VLDL represents the sum of cholesterol concentrations from fraction 2 to 7 (VLDL fractions); LDL represents the sum of cholesterol concentrations from fraction 8 to 14 (LDL fractions); HDL represents the sum of cholesterol concentrations from fraction 15 to 22 (HDL fractions) (B). Values are means ± SEM (10 mice per group). *P<0.05; **P<0.01; ***P<0.001; ns, not significant.
LXR agonist regulated hepatic gene expression in ApoE<sup>−/−</sup> mice reconstituted with C57BL/6 bone marrow

As expected, LXR agonist treatment increased the liver weight (data not shown) and strongly up-regulated the hepatic expression of LXR target genes SREBP-1c, FAS, and LPL compared to mice fed chow diet alone (Figure 4A, 4B). This LXR agonist-induced hepatic lipogenesis has been well established as a positive control for LXR activation in mice<sup>31,32</sup>.

After transplanting C57BL/6 bone marrow into ApoE<sup>−/−</sup> mice, the mRNA expression of apoE showed up in the liver. LXR agonist treatment further up-regulated the hepatic gene expression of apoE compared to group fed chow diet alone (2-fold, \( P<0.001 \) in initial lesions; 1.7-fold, \( P<0.001 \) in advanced lesions) (Figure 5A, 5D). In addition, LXR agonist treatment significantly up-regulated the hepatic expression of ABCG5 and ABCG8 compared to group fed chow diet alone (Figure 5B, 5E), suggesting promoted biliary cholesterol secretion upon LXR activation.

In line with the significantly elevated plasma HDL-cholesterol level in ApoE<sup>−/−</sup> mice reconstituted with C57BL/6 bone marrow, LXR agonist treatment significantly increased the hepatic expression of ABCG1 (1.4-fold, \( P<0.05 \) in initial lesions; 2.3-fold, \( P<0.001 \) in advanced lesions) and ApoA-I (1.6-fold, \( P<0.001 \) in advanced lesions) compared to the chow group (Figure 5C, 5F), suggesting that LXR activation promoted the cholesterol efflux capacity.

![Figure 4](image_url)

**Figure 4.** Changes in hepatic gene expression profiles during regression studies in initial lesions (A) and advanced lesions (B). Total RNA was extracted from liver, and relative mRNA expression of SREBP-1c, FAS, CYP7A1, and LPL were determined by quantitative PCR and presented as fold-change relative to baseline group (C). Values are means ± SEM (10 mice per group). *\( P<0.05 \); **\( P<0.01 \); ***\( P<0.001 \); ns, not significant.
Figure 5. Changes in hepatic gene expression profiles during regression studies in initial lesions (A, B, C) and advanced lesions (D, E, F). Total RNA was extracted from liver, and relative mRNA expression of ApoE, ABCG5, ABCG8, ApoA-I, and ABCG1 were determined by quantitative PCR and presented as fold-change relative to baseline group (C). Values are means ± SEM (10 mice per group). *P<0.05; **P<0.01; ***P<0.001; ns, not significant.

Atherosclerotic plaque regression after transplanting wild-type C57BL/6 bone marrow into ApoE−/− mice
With WTD feeding, ApoE−/− mice developed atherosclerotic lesions of ± 70x10³ μm² at the aortic root in initial lesion study (Figure 6A) and ± 550x10³ μm² in advanced lesion study at baseline (Figure 6B). In control mice reconstituted with ApoE−/− bone marrow, plaque size did not decrease after 6 weeks of chow diet feeding, with or without LXR agonist supplementation (Figure 6B). In contrast, six weeks after transplanting wildtype C57BL/6 bone marrow into ApoE−/− mice, with chow diet feeding alone, the lesion size decreased significantly to ± 39x10³ μm² (-45%,
LXR agonist reduced plaque macrophage content during atherosclerosis regression

Further analysis of atherosclerotic lesion composition showed that the collagen content of the plaque in initial lesions decreased after bone marrow reconstitution with chow diet alone (-66%, \( P<0.001 \)), and LXR agonists further reduced the collagen amount in initial plaques (-81%, \( P<0.001 \) compared to Baseline; -45%, \( P<0.01 \) compared to chow group) (Figure 7A). However, in advanced plaques, despite the significant reduction of plaque size after BMT, the collagen content of plaque in ApoE\(^{-/-}\) mice with wildtype C57BL/6 bone marrow remained the same, with or without treatment of LXR agonist (Figure 7B). In contrast, the absolute macrophage-positive area and percentile of area of the lesion occupied by macrophages decreased dramatically in accordance to the reduction of total lesion size. In the initial lesion induced by 3 days of WTD feeding, the size of macrophage-positive area was 63x10\(^3\) \( \mu \text{m}^2 \) at aortic root as baseline (Figure 8A). In the advanced lesion induced by 6 weeks of WTD feeding, the size of macrophage-positive area was 125x10\(^3\) \( \mu \text{m}^2 \) at aortic root as baseline (Figure 8B). Six weeks after transplanting wildtype C57BL/6 bone marrow into ApoE\(^{-/-}\) mice, the macrophage-positive area decreased significantly in both studies with chow diet alone (-43%, \( P<0.05 \) in initial lesions; -96%, \( P<0.01 \) in advanced lesions; Figure 8A, 8B). LXR agonist treatment further reduced the plaque macrophage content significantly in both initial lesions (-70%, \( P<0.001 \) compared to Baseline; -50%, \( P<0.01 \) compared to chow group) and in advanced lesions (-100%, \( P<0.01 \) compared to Baseline; -100%, \( P<0.01 \) compared to chow group) (Figure 8A, 8B) that no positive macrophage-staining was visible anymore. The results indicated that the reduction in plaque size observed in this mouse model was primarily due to the decreased macrophage content in the plaques.

Figure 6. Changes in atherosclerotic lesion size during regression studies in initial lesions (A) and advanced lesions (B), and comparison of lesion size from both studies expressed as percentage of baseline lesion (C). Cryostat sections of the aortic root in heart were stained with oil-red O to identify lipids, and the lesion size was quantified. Values are means \( \pm \) SEM (10 mice per group). **\( P<0.01 \); ***\( P<0.001 \); ns, not significant.
Figure 7. Changes in size of collagen area in atherosclerotic plaques during regression studies in initial lesions (A) and advanced lesions (B) were measured. Cryostat sections of the aortic root in heart were stained with Masson's Trichrome Stain kit and counterstained with hematoxylin to assess the extent of collagen deposition and the structural integrity of fibrillar collagen in the plaque. Size of collagen area in plaques was quantified. Values are means ± SEM (10 mice per group). **P<0.01; ***P<0.001.

Figure 8. Changes in size of macrophage-positive area in atherosclerotic plaques during regression studies in initial lesions (A) and advanced lesions (B) were measured. Cryostat sections of the aortic root in heart were stained with MOMA-2 antibody (rat antibody directed against murine monocytes/macrophages) and rabbit anti rat IgG/HRP to identify macrophage-positive area. Sections were developed using NovaRED Peroxidase Substrate Kit, and the size of macrophage-positive area was quantified. Values are means ± SEM (10 mice per group). *P<0.05; **P<0.01; ***P<0.001; ns, not significant.
In the current study, we set up a new mouse model to study atherosclerotic lesion regression. Our results show that ApoE\(^{-/-}\) mice reconstituted with bone marrow from wildtype C57BL/6 mice form a promising model to induce rapidly normalized plasma lipoprotein profiles and the regression of pre-existing atherosclerotic plaques.

In previous studies where dramatic regression of large advanced lesions was achieved with or without LXR agonist\(^{20,21}\), surgical aorta transplantation into wildtype mice was performed to rapidly improve the atherosclerotic plaque milieu. In our study, we used the BMT procedure to induce plaque regression, which raises the opportunity to analyze the relative importance of individual genes in hematopoietic cells for plaque regression, by using specific knockout mice as bone marrow donors.

Bone marrow transplantation leads to the replacement of bone marrow-derived cells, including recipient tissue macrophages by cells of donor origin. After transplantation of bone marrow from mice with wildtype apoE expression into ApoE\(^{-/-}\) mice, we observed rapid improvement and normalization of the plasma cholesterol profile. This cholesterol lowering effect of macrophage-derived apoE was reported in previous studies, where apoE of donor origin was present in the recipient peripheral circulation as early as 2 weeks after transplantation, and by 4 weeks, apoE production by bone marrow-derived cells was sufficient to normalize plasma lipid levels of ApoE\(^{-/-}\) recipient mice\(^{33,34}\). The apoE level in circulation after BMT was only 12.5% of those in wildtype mice but nevertheless sufficient to reduce the severe hypercholesterolemia of ApoE\(^{-/-}\) mice, due to accelerated hepatic clearance of plasma cholesterol and promoted cholesterol efflux\(^{19,35,36}\).

Raffai et al for the first time addressed the apoE-mediated mechanisms of atherosclerosis regression\(^{37}\). They demonstrate that apoE promotes the regression of atherosclerosis independently of lowering plasma cholesterol levels. In contrast, a study from Shi et al concluded differently. They demonstrated that regression of atherosclerotic lesions is a slow process and macrophage-derived apoE was insufficient to induce significant regression of established atherosclerotic lesions in ApoE\(^{-/-}\) mice, although it was sufficient to eliminate hypercholesterolemia and prevent progression of aortic lesions\(^{34}\). The difference in conclusions between their study and our current observations may come from the differences in experimental set-up.

Study from Zhao et al showed that in both LDLr\(^{-/-}\) and C57BL/6 mice, switch of an atherogenic diet to regular chow diet could not trigger lesion regression despite a rapid and dramatic reduction in plasma total cholesterol levels. In LDLr\(^{-/-}\) mice, lesion sizes even increased despite the cholesterol lowering\(^{38}\). Similar observations were reported where switching high-fat atherogenic diet to a standard chow diet led to markedly reduced plasma (V)LDL-cholesterol without significant reduction in lesion size\(^{37}\), or with even significantly increased lesion size\(^{39}\). In the current study with BMT procedure, chow diet alone induced a significant regression of both initial and advanced plaques, which was in accordance with study from Bengtsson et al where ApoE\(^{-/-}\) mice were transplanted with wildtype bone marrow and a 35% plaque regression was observed\(^{40}\). Taken together, our data confirmed that ApoE\(^{-/-}\) mice reconstituted with wildtype C57BL/6 bone marrow is a valid regression mouse model.
We performed this regression study in parallel on initial and advanced lesions. The atherosclerotic plaque is a dynamic tissue, where increases in cell number (driven by cell proliferation and migration) and decreases in cell number (driven by cell death and possibly emigration) are continuous processes. Initial atherosclerotic lesions are primarily composed of lipid-loaded macrophages. Stable advanced lesion contains a macrophage core, a small necrotic core, if present at all, extracellular matrix and a firm fibrous cap of smooth muscle cells (SMCs). Instable advanced atherosclerotic lesions are characterized by a thin fibrous cap containing few SMCs and overlying a large necrotic core composed of dead cells, lipid deposits, and cellular debris. For long it has been thought that advanced lesion could not regress since they contain thick fibrous cap, large amount of necrotic material, extracellular lipids and extracellular matrix. In the current study, we showed that via the bone marrow transplantation technique, successful plaque regression can be induced in both initial and advanced lesion. The current mouse model is thus a good model to study atherosclerosis regression in different stages of the disease.

LXR agonists have potent anti-atherogenic effects in different hyperlipidemic mouse models. Several studies have demonstrated that activation of LXR significantly up-regulated cholesterol efflux activity and inhibited the development of atherosclerosis. However, the ability of LXR agonists to abrogate pre-existing cardiovascular disease by inducing regression and stabilization of established atherosclerotic lesions has not been widely addressed. We used LXR agonist treatment in our mouse model to assess the potential of LXR activation to induce atherosclerosis regression. The LXR agonist T0901317 not only reduced plasma (V)LDL-cholesterol levels, but also significantly increased HDL-cholesterol. This indicates that LXR agonist, supplemented in chow diet, rapidly optimizes the plasma lipoprotein profile and achieves a regressive plasma environment in this mouse model. In addition, compared to group fed with chow diet alone, LXR agonist treatment in this study induced further 48% reduction in initial lesion size, 17% reduction in advanced lesion size, and also a further 50% reduction in the macrophage-positive area size in initial lesions. In advanced lesions, there was merely visible macrophage-positive staining observed in LXR agonists treated group, indicating the diminishing of macrophages during atherosclerotic regression in our mouse model. Combined, our results were in line with findings from Feig et al. that LXR activation is necessary for maximal effects on plaque macrophage content reduction during atherosclerosis regression in mice.

To further analyze the dynamics and the cellular cause of the rapid reduction of total plaque size, we examined the lesion composition in detail. The atherosclerotic plaque is a complicated structure. In addition to cholesterol-filled macrophage core, it contains large numbers of immune cells, SMCs, vascular endothelial cells, and a large amount of extracellular matrix products that includes sulfated glycosaminoglycans, collagen, fibrin, and extracellular lipids. The complexity of atherosclerosis is highlighted by the multifaceted effects that apoptosis and proliferation of specific cell types can have on vessels at different stages of the disease. In initial lesions, a 45% reduction in lesion size was observed with chow diet alone, and even a 71% reduction with LXR agonist treatment, whilst in advanced lesions there was only a 23% reduction with chow diet alone, and a 36% reduction with LXR activation. This can be explained by the difference in plaque composition and characteristics between initial and advanced lesion. Initial lesions
contain primarily cholesterol-filled macrophages, while in advanced lesion, SMCs and extracellular matrix products comprise the major structural components of the atherosclerotic plaques. SMCs and extracellular matrix products remained unchanged and are more difficult to be modulated during the regression process. Therefore, the extent of lesion size reduction was smaller in advanced lesions compared to initial lesions.

Interestingly, our lesion composition analysis showed that the reduction of total lesion size during regression was primarily due to the reduction and diminishing of macrophage content in the plaque. The fate of the macrophages during lesion regression is currently under debate. It has been proposed that regression is not merely a rewinding of progression, but instead involves induction of CCR7 expression, a mediator of leukocyte emigration, in foam cells and emigration of the maladaptive macrophage infiltrate, followed by the initiation of influx of healthy phagocytes that mobilize necrotic debris and all other components of advanced plaques. Interestingly, Ye et al showed that macrophage infiltration into pre-existing advanced lesions was limited, likely because of the formation of fibrous caps. In contrast, Potteaux et al also showed that regression of atherosclerosis after apoE complementation in ApoE−/− mice did not involve migratory egress of macrophages from plaques or induction of CCR7. Instead, marked suppression of monocyte recruitment coupled with a stable rate of apoptosis accounted for loss of plaque macrophages, suggesting that therapies to inhibit monocyte recruitment to plaques may constitute a viable strategy to reduce plaque macrophage burden than attempts to promote migratory egress. Further research is necessary to establish the processes and mechanisms underlying the diminished macrophage content during regression in our experimental mouse model.

In conclusion, our current study shows that 1) ApoE−/− mice reconstituted with bone marrow from C57BL/6 mice represents a promising mouse model with chow diet feeding to study atherosclerosis regression, providing an alternative model to investigate plaque regression; and 2) rapidly optimized plasma lipoprotein profiles, combined with LXR agonist treatment, induced favorable gene expression profiles that can induce significant regression of both initial and more advanced atherosclerotic plaques.

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