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The interplay between cholesterol and inflammation in the evolution of atherosclerosis

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Citation

Verschuren, L. (2009, January 22). *The interplay between cholesterol and inflammation in the evolution of atherosclerosis*. Retrieved from <https://hdl.handle.net/1887/13415>

Version: Corrected Publisher's Version

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Chapter 6

LXR agonist suppresses atherosclerotic lesion growth and promotes lesion regression in ApoE*3Leiden mice: time course and potential mechanisms

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Journal of Lipid Research, in press

Abstract

Background: The aim of this study was to define the anti-atherosclerotic role of liver-X-receptors (LXRs) under both lesion progressive and lesion regressive conditions, to establish a temporal line of events, and to gain insights into the mechanisms underlying the anti-atherogenic potency of LXRs.

Methods and results We used ApoE*3Leiden mice to comprehensively and time-dependently dissect how T0901317, an LXR-agonist, inhibits initiation and progression of atherosclerotic lesions, and regresses existing lipid- and macrophage-rich lesions. T0901317 strongly suppresses lesion evolution and promotes lesion regression regarding lesion number, area, and severity. Quantitative plasma and vessel wall analyses corroborated by immunohistochemical evaluation of the aortic lesions revealed that under progressive (high-cholesterol diet) as well as regressive (cholesterol-free diet) conditions T0901317 (i) significantly increases plasma triglyceride and total cholesterol levels; (ii) does not affect the systemic inflammation marker, SAA; (iii) suppresses endothelial monocyte adhesion; and (iv) induces the expression of the cholesterol efflux-related genes ApoE, ABCA1 and ABCG1. Furthermore, under progressive conditions, T0901317 suppresses the vascular inflammatory status (NF- κ B) and the vascular expression of adhesion molecules (E-selectin, ICAM-1, and CD44), lowers lesional macrophage accumulation, and blocks lesion evolution at the transition from lesional stage II to III. Under regressive conditions, T0901317 induces lesional macrophage disappearance and increases the expression of the chemokine receptor CCR7, a factor functionally required for regression.

Conclusions The LXR-agonist T0901317 retards vascular lesion development and promotes lesion regression at several levels. The findings support that vascular LXR is a potential anti-atherosclerotic target.

Introduction

Coronary atherosclerosis represents the leading cause of morbidity and mortality of men and women throughout the Western world. Hypercholesterolemia is a well-established risk factor for the incidence of atherosclerosis and its pathologic complications¹. However, despite the success of cholesterol-lowering statins in reducing cardiovascular causes of death, two thirds of the statin-treated patients still experience cardiovascular events. Consequently, focus has switched to risk factors other than hypercholesterolemia.

Atherosclerosis is now recognized as a multifactorial, multistep disease with numerous etiologies which have to act in concert to initiate and promote the atherosclerotic process. In addition to traditional risk factors such as hypertriglyceridemia, low high-density lipoprotein (HDL), and hypertension, inflammation is accepted as a major driving force of atherosclerotic lesion development. Inflammation has a key role in lesion initiation and evolution, and encompasses both cellular and molecular components².

Liver-X-receptor (LXR) belongs to the nuclear hormone receptor superfamily of ligand-activated transcription factors. Two LXR isoforms have been described so far, LXR α and LXR β . LXR β has a ubiquitous tissue distribution, whereas LXR α predominates in liver, adipose tissue, intestinal tissue, and macrophages; both isoforms respond to the same natural and synthetic ligands³. LXRs control genes involved in intestinal cholesterol absorption, hepatic bile acid synthesis, cholesterol efflux, vascular foam cell formation, and inflammation⁴. These properties make LXRs a potential target for therapeutic intervention in the atherosclerotic process. Studies performed over the last several years have established that LXRs are anti-atherosclerotic factors owing to their ability to regulate cholesterol and lipid homeostasis and to inhibit inflammation within the arterial wall⁵⁻⁸. However, these studies mainly focused on lesion growth but not lesion regression, did not establish a temporal line of events, and only partly addressed the mechanisms underlying the anti-atherogenic potency of LXRs.

Here we describe the impact of an LXR-agonist, T0901317, on the progression and regression of atherosclerosis in transgenic ApoE*3Leiden (E3L) mice, a well-established mouse model for atherosclerosis.^{9, 10} E3L mice are highly responsive to cholesterol-containing diets, resulting in strongly elevated plasma cholesterol and triglyceride levels, with a prominent increase in VLDL- and LDL-sized lipoprotein particles. E3L mice do not develop atherosclerosis on a regular chow diet, but atherosclerosis in E3L mice can be initiated by feeding of a cholesterol-containing, Western type diet⁹, whereas regression of pre-existing lesions can be induced using cholesterol-depleted diets¹¹. E3L mice respond

to hypolipidemic drugs with cholesterol-lowering and appear useful in predicting effects of pharmaceutical modifiers in humans⁹. Our study confirms the results previously published by other groups in different mouse models (ApoE^{-/-} or Ldlr^{-/-} mice) regarding some of the effects of LXR-agonists on lesion development, but adds some important novel information with regard to the action of the LXR-agonist on the vasculature, in particular to vascular inflammation, adhesion molecule expression and under regressive conditions, potential mechanisms involved in macrophage disappearance. Also, the analysis of the experiments at different time points allowed establishing a temporal line of events.

Methods

Animals

Female ApoE*3Leiden transgenic (E3L) mice (TNO-Pharma, Gaubius Laboratory, Leiden, The Netherlands) were characterized for expression of human ApoE by enzyme-linked immunosorbent assay (ELISA). The animals were housed in wire-topped Macrolon cages with a layer of sawdust as bedding, and diets and water were given *ad libitum*.

T0901317-Atherosclerosis studies

Progression studies. To establish a time course of events, three separate progression studies were performed, with mice (age 10-12 weeks at start of experiment) sacrificed after t=2 weeks, t=10 weeks, or t=15 weeks of experimental treatment, respectively.

Study 1 (main study; sacrifice at 10 weeks). During a run-in period of 3 weeks, 24 female E3L mice received an atherogenic, 1% (w/w) cholesterol-containing Western-type diet (Hope Farms, Woerden, The Netherlands), further referred to as high-cholesterol (HC) diet. Then, mice were subdivided into 2 experimental groups (n=12) and matched for plasma cholesterol and triglycerides. In one group, HC was continued for another 10 weeks (HC group). The other, T0901317-treated group (HC+T0901317 group) received HC supplemented with 0.01% w/w T0901317 (Sigma Aldrich). Based on food intake in the HC+T0901317 group, the daily dose of T0901317 was 11 mg/kg bodyweight.

Study 2: as study 1, but sacrifice at 15 weeks; n=15 per group.

Study 3: (short study; sacrifice at 2 weeks). E3L mice were treated with HC-diet supplemented with or without 0.01%(w/w) T0901317 (n=8 per group; no run-in) and were sacrificed after 2 weeks of treatment.

Regression study. Twenty-six female E3L mice received HC for 18 weeks to develop atherosclerotic lesions. Then, 10 mice were sacrificed for atherosclerotic lesion analysis. The 16 remaining mice were subdivided into two groups of 8 mice each and switched for 8 weeks to a regressive, cholesterol-depleted diet (RD), supplemented with 0.01% (w/w) T0901317 (RD+T0901317 group) or not (RD group).

Analysis of plasma lipids, lipoproteins and plasma inflammation markers

Total plasma cholesterol and triglyceride levels were measured after 4 hours of fasting, using kits No. 11489437 and 11488872 (Roche Diagnostics, Almere, The Netherlands), respectively. For lipoprotein profiles, pooled plasma was fractionated using an ÅKTA FPLC system (Pharmacia, Roosendaal, The

Netherlands).¹² The plasma levels of E-selectin (R&D Systems) and SAA (Biosource) were determined by ELISA as reported.¹³

Atherosclerotic lesion analysis

At the end of a treatment period, mice were euthanized to collect hearts and aortas. Hearts were fixed and embedded in paraffin to prepare serial cross sections (5 μ m-thick)

throughout the entire aortic root area for (immuno)histological analysis.^{12, 14} Cross-sections were stained with hematoxylin-phloxine-saffron and atherosclerosis was analyzed blindly in 4 cross-sections of each specimen (at intervals of 40 μ m). QWin-software (Leica)^{12, 14} was used for morphometric computer-assisted analysis of lesion number, lesion area and lesion severity according to the classification of the American Heart Association¹⁵ as reported.^{12, 14}

Monocyte adhesion and macrophage area were determined essentially as described previously.^{14, 16, 17} For immunostaining of ICAM, LXR α/β , p50-NF- κ B, p65-NF- κ B, CD44 and CCR7 antibodies GTX76543 (Genetex), sc-1000, sc-114, sc-109, sc-18849 and sc-9701 (Santa Cruz Biotechnology) were used, respectively.¹⁸

Nucleic acid extraction and Real-time PCR

Total RNA was extracted from individual aortas (n=5 per group) using RNeasy (Qiagen, Crawley, The Netherlands) and glass beads according to the manufacturer's instructions. The integrity of each RNA sample obtained was examined by Agilent Lab-on-a-chip technology using the RNA 6000 Nano LabChip kit and a bioanalyzer 2100 (Agilent Technologies, Amstelveen, The Netherlands). Then, cDNA was prepared using kit #A3500 (Promega, Leiden, The Netherlands) for real-time polymerase chain reaction (RT-PCR) analysis. Mastermix (Eurogentec, Seraing, Belgium), the ABI-7700 system (Perkin-Elmer Biosystems, Nieuwekerk a/d IJssel, the Netherlands), and established primer/probe sets were used according to the manufacturer's instructions. Cyclophilin A (PE Biosystems) was used as a reference.

Statistical methods

Significance of difference was calculated by analysis of variance (ANOVA) test followed by a least significant difference post hoc analysis using SPSS 11.5 for Windows (SPSS, Chicago, USA). The level of statistical significance was set at $P < 0.05$.

	HC	HC+ T0901317
Average food intake (g/day)	2.5 \pm 0.2	2.2 \pm 0.2*
Average body weight (g)	20.8 \pm 0.4	19.6 \pm 0.5
Average total cholesterol level (mM)	18.7 \pm 3.6	23.4 \pm 4.1*
Average triglyceride level (mM)	1.8 \pm 0.4	12.3 \pm 4.4*

Table 1. Metabolic parameters of control and T0901317-treated, cholesterol-fed E3L mice. Data are expressed as means \pm SD; n=12 for each group; * $P < 0.05$ vs. HC

Results

T0901317 enhances plasma lipid levels in E3L mice

Food intake of mice fed HC+T0901317 for ten weeks was slightly lower than that in animals on HC (2.2 ± 0.2 grams per day (g/day) vs. 2.5 ± 0.2 g/day; $P < 0.05$; Table 1). At the end of the treatment period, there was no significant difference in bodyweight between the two groups (HC+T0901317, 19.6 ± 0.5 g; HC, 20.8 ± 0.4 g; Table 1). Plasma cholesterol levels in HC+T0901317 (23.4 ± 4.1 mM) were significantly increased compared with HC (18.7 ± 3.6 mM, $P < 0.05$; Table 1). This rise in plasma total cholesterol levels in HC+T0901317 can be mainly ascribed to a particular subfraction (fractions 10-17; Figure 1A), co-eluting with particles in the IDL/LDL range. T0901317 strongly (6.8 times) increased fasting plasma triglyceride levels (HC+T0901317, 12.3 ± 4.4 mM; HC, 1.8 ± 0.4 mM; $P < 0.05$; Table 1), which rise is entirely confined to particles in the VLDL-range (Figure 1B). Quantitative RT-PCR analysis of liver samples indicates that the strong increase in triglyceride levels in the T0901317-treated group may at least partly be explained by the 2.2-fold ($P < 0.01$) elevated expression of the lipogenic gene, sterol regulatory binding protein (SREBP)-1c.

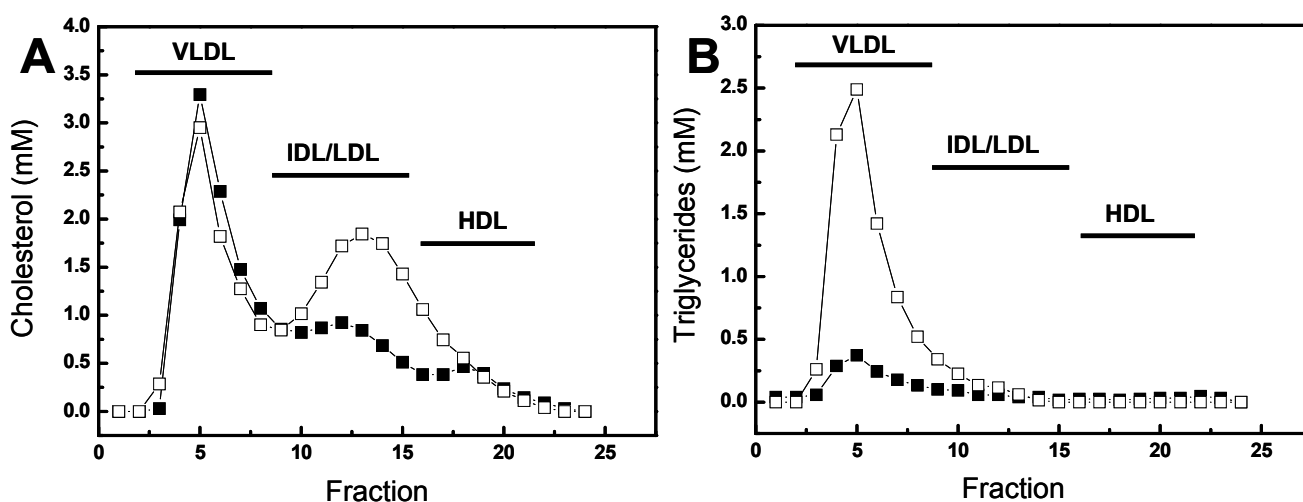


Figure 1: Lipoprotein profiles of control and T0901317-treated, cholesterol-fed E3L mice. **(A)**, Cholesterol content in fractionated plasma samples. **(B)**, Triglyceride content in fractionated plasma samples. Solid squares indicate HC, open squares indicate HC+T0901317.

T0901317 reduces atherosclerotic lesion number, size and severity

After 10 weeks of experimental treatment, *early* atherosclerotic lesion formation was analyzed in cross-sections of the aortic valve area. The average lesion number per mouse was 18.3 ± 2.3 in HC and reduced by 64% to 6.5 ± 1.3 in HC+T0901317 ($P < 0.01$; Figure 2A). Measurement of the total cross-sectional lesion area showed a similar picture (Figure

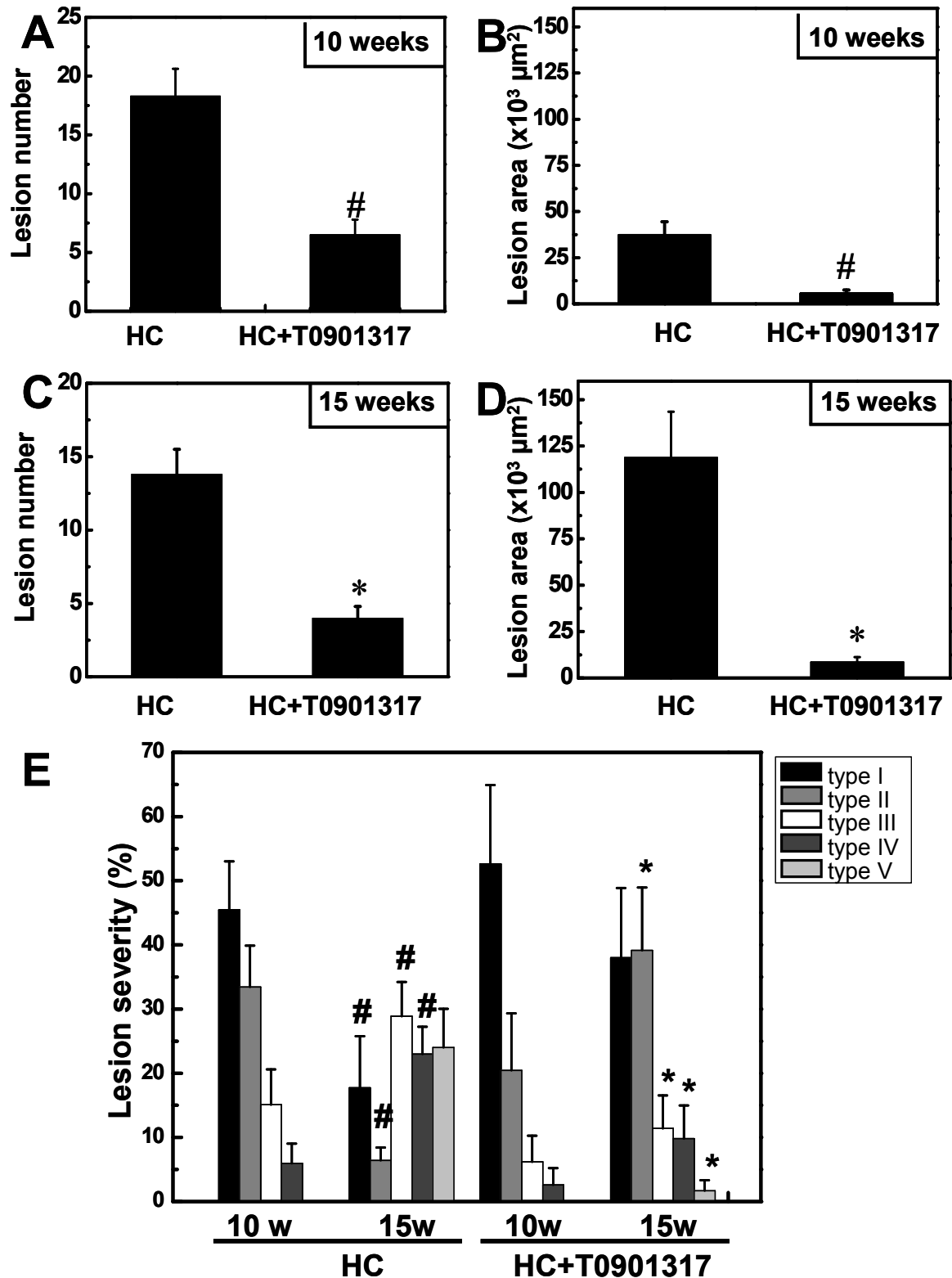


Figure 2: Effect of T0901317 on atherosclerosis development in aortic valve area. Shown are effects of T0901317 on total lesion number after 10 weeks (A) and 15 weeks (C) of treatment and effects of T0901317 on total cross-sectional lesion area after 10 weeks and 15 weeks (D) of treatment. The effect of T0901317 on lesion severity is presented as percentage of cross-sections analyzed (E). Data represent mean values \pm SEM ($n \geq 10$ mice per group) # $P < 0.05$ vs. HC-10 weeks, * $P < 0.05$ vs. HC-15 weeks.

2B): HC displayed a cross-sectional lesion area of $37027 \pm 7450 \mu\text{m}^2$ and HC+T0901317 reduced the lesion area by 85% to $5484 \pm 2019 \mu\text{m}^2$ ($P < 0.05$).

To assess whether these differences merely reflect a difference in time of onset of the disease process or are the result of diminished lesion initiation and progression, lesion number and size were also analyzed (in a separate experiment) after a 15-week-treatment. The difference in lesion number of the two groups resembled that of the 10-week experiment (Figure 2C), while the lesion area was substantially increased in HC (3.2 fold), but only marginally (1.5 fold) in HC+T0901317 after 15 weeks (Figure 2D), indicating that T0901317 is interfering with both lesion initiation and lesion development rather than retarding the onset of the disease process.

	t=0	HC	HC+ T0901317
Serum Amyloid A ($\mu\text{g/ml}$)	10.1 ± 4.6	$21.7 \pm 15.3^{\#}$	$24.5 \pm 12.1^{\#}$
E-Selectin (ng/ml)	84.4 ± 9.7	$97.2 \pm 13.7^{\#}$	$84.4 \pm 12.2^*$

Table 2. Plasma inflammation markers of control and T0901317-treated, cholesterol-fed E3L mice. Shown are average plasma levels of Serum Amyloid A and E-Selectin at the start (t=0) and the end (t=10 weeks) treatment period (means \pm SD n=12 for each group; $^{\#}P < 0.05$ vs. t=0 and $^*P < 0.05$ vs. HC).

This conclusion was further strengthened by evaluating lesion severity. Cross-sections of the aortic root were morphologically analyzed and lesions were graded according to the classification of the American Heart Association.¹⁵ Grading after 10 weeks demonstrated that lesions from both HC and HC+T0901317 mainly contained mild type I/II lesions (~80% of all classified lesions) and hardly type IV/V lesions (Figure 2E). After 15 weeks, a clear shift in lesion severity was observed in HC but not in HC+T0901317. In HC, $24\% \pm 6\%$ of all cross-sections contained type I/II lesions and $76\% \pm 5\%$ contained type III/IV/V lesions (Figure 2E). In contrast, in HC+T0901317 treated mice $77\% \pm 10\%$ of all cross-sections contained type I/II lesions and $23\% \pm 4\%$ contained type III/IV/V lesions, thereby remarkably resembling the findings obtained after the 10-week-treatment. Importantly, T0901317 blocked lesion progression at the transition from lesion severity stage II to III, which is characterized by the absence of smooth muscle cell (SMC) proliferation and migration to the cap, a hallmark of type III lesions.

Thus, T0901317 strongly suppresses atherogenesis, both at the initiation and the progression phase of disease. The question therefore arises by what mechanism T0901317 interferes with the atherosclerotic process.

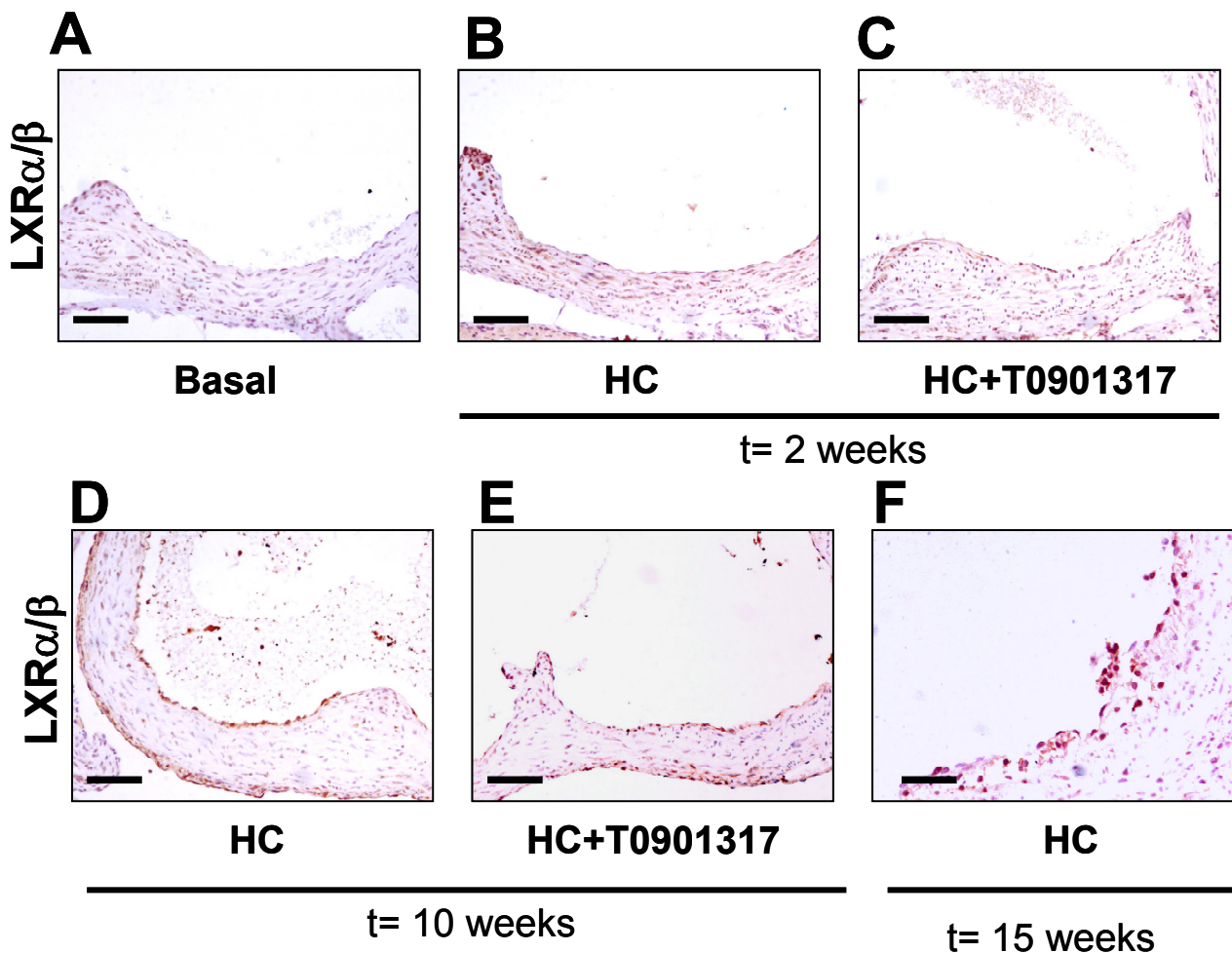


Figure 3: Vascular LXR α/β expression is induced in cholesterol-fed E3L mice. Representative photomicrographs of immunohistochemical staining of LXR α/β -immunoreactivity (IR) in cross-sections of the aortic root: in basal chow-fed E3L mice (A); after 2 weeks of HC (B) or HC+T0901317 (C); after 10 weeks of HC (D) or HC+T0901317 (E); after 15 weeks of HC (F). Bar= 50 μ m.

The vessel wall as target for T0901317

To evaluate whether the anti-atherosclerotic effects of T0901317 are paralleled by anti-inflammatory effects, we analyzed systemic inflammatory markers originating from liver (SAA) or vasculature (E-selectin). HC markedly and significantly elevated plasma levels of both SAA (2.1-fold) and E-selectin (1.2-fold) (Table 2). T0901317 significantly suppressed HC-induced E-selectin but not SAA plasma levels, pointing to a direct anti-inflammatory effect of T0901317 on the vascular endothelium. To seek further evidence for a direct effect of T0901317 on the vessel wall we evaluated vascular expression of LXR α/β immunohistochemically using an antibody recognizing both LXR isoforms (see Methods section). In chow-fed E3L mice no LXR α/β expression was detectable in the vasculature (Figure 3A), but 2 weeks after starting the HC diet, expression of LXR α/β was demonstrable in both the HC and HC+ T0901317 group (Figure 3B-C) and remained so for

the rest of the experimental treatment period (Figure 3D-E). In both experimental groups expression of LXR α/β was mainly confined to the endothelium and to a lesser extent to SMC; no notable difference in staining intensity was seen between the two groups (Figure 3B-E). Upon lesion progression, accumulating monocyte-derived macrophages in the lesions also stained positive for LXR α/β (Figure 3F). QRT-PCR analysis revealed that both LXR α and LXR β were about two-fold up-regulated by dietary cholesterol.

Together, these data demonstrate, for the first time, that dietary cholesterol induces LXR protein expression in the vasculature (endothelium, SMC, monocyte-derived macrophages), thus enabling T0901317 to specifically suppress HC-induced expression of endothelial E-selectin and thereby to lower plasma levels of E-selectin.

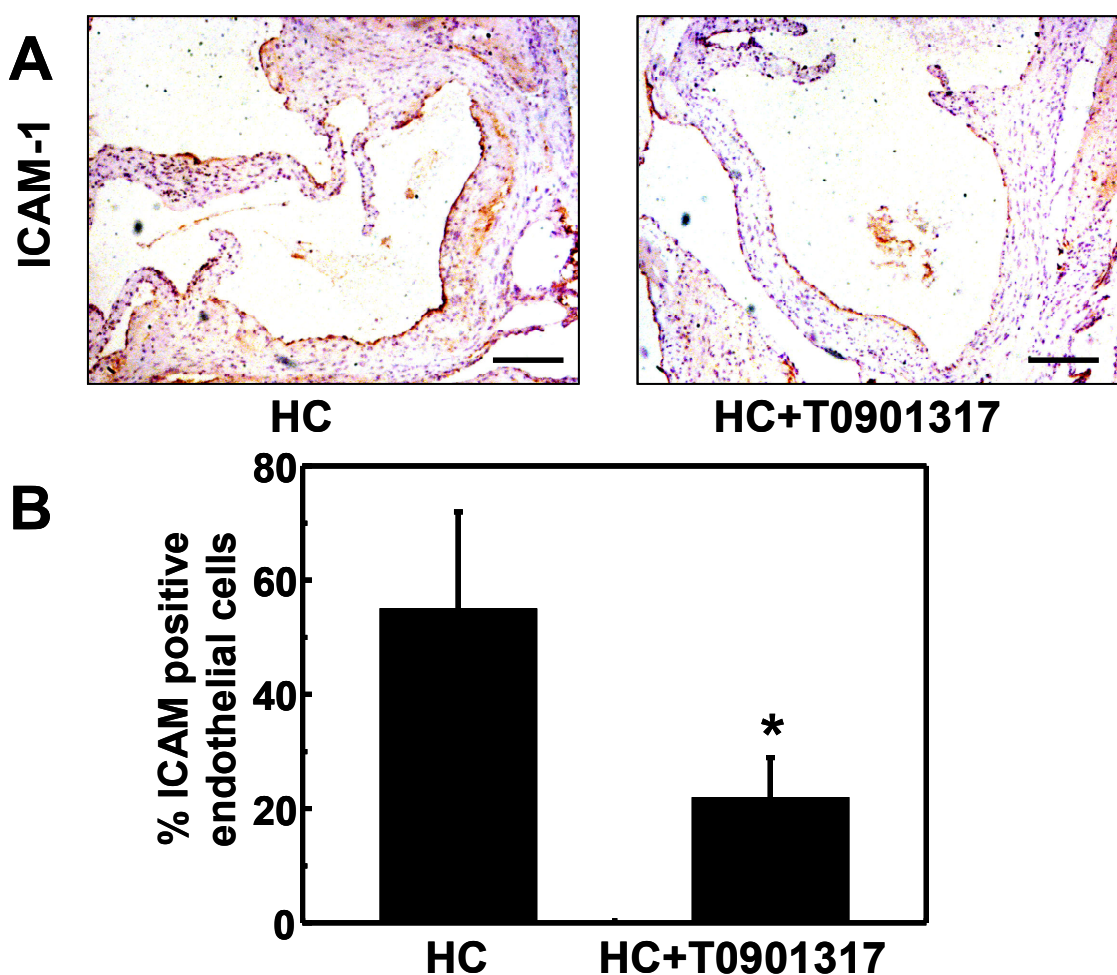


Figure 4: Effect of T0901317 on vascular ICAM-1 and NF- κ B expression. Representative photomicrographs of immunohistochemical staining of ICAM-1-IR (A) after 10 weeks of treatment with HC or HC+T0901317. Analyzed percentage of ICAM-1-IR positive EC in control vs treatment group (B), (*P<0.05 vs. HC). Bar= 100 μ m. (Figure 4 is continued on next page)

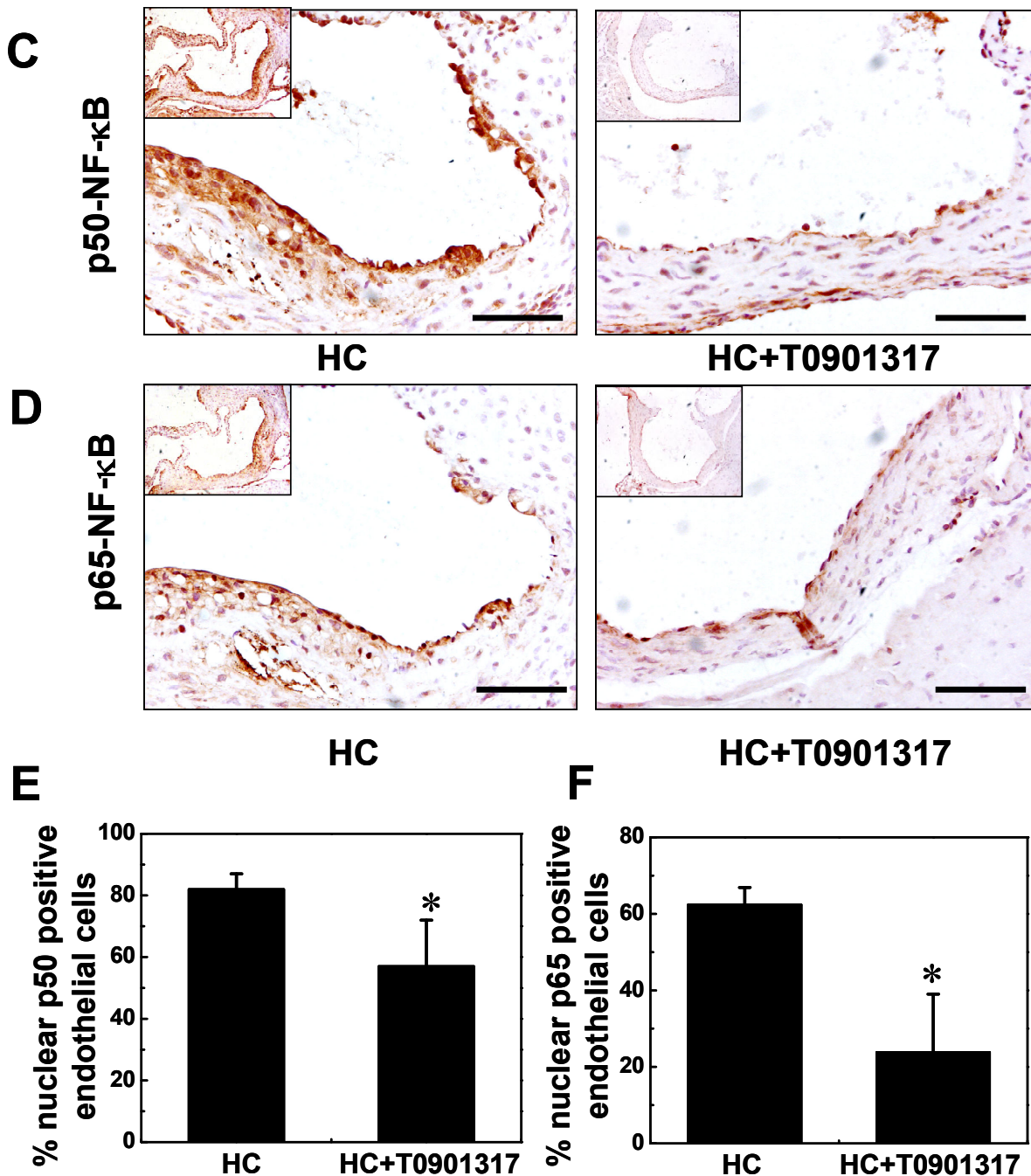


Figure 4. Effect of T0901317 on vascular ICAM-1 and NF-κB expression. (continued) Representative photomicrographs of immunohistochemical staining of p50-NF-κB-IR (C), and p65-NF-κB-IR (D) after 10 weeks of treatment with HC or HC+T0901317. (* $P < 0.05$ vs. HC). Bar= 100 μ m. Analyzed percentage of nuclear p50-NF-κB-IR positive EC (E), and nuclear p65-NF-κB-IR positive EC (F) after 10 weeks of treatment with HC or HC+T0901317.

Anti-atherogenic action of T0901317 in the vasculature

The anti-inflammatory effect of T0901317 in the vasculature is not restricted to E-selectin. Immunohistochemical staining of ICAM-1 underlines that T0901317 suppresses endothelial adhesion molecule expression more generally. Whereas in HC 55% \pm 17% of the aortic endothelial cells (EC) expressed ICAM-1, in HC+T0901317 only 22% \pm 7%

($P < 0.05$) of the cells did so (Figures 4A and 4B). Furthermore, the total number of EC per cross-section with positive p65-NF- κ B- or p50-NF- κ B-staining (in cytosol and/or nucleus) was strongly reduced in HC+T0901317 (Figure 4C-D). Also, the number of EC showing nucleus-associated (i.e. active) p65-NF- κ B or p50-NF- κ B immunoreactivity was strongly and significantly ($P < 0.05$) reduced in HC+T0901317 compared with HC (Figure 4E-F). These results are further evidence that T0901317 not only lessens the number of NF- κ B-positive EC, but also the number of active NF- κ B-containing EC, thus providing a rationale for the reduced expression of cellular adhesion molecules and the overall suppressive effect on lesion initiation with T0901317.

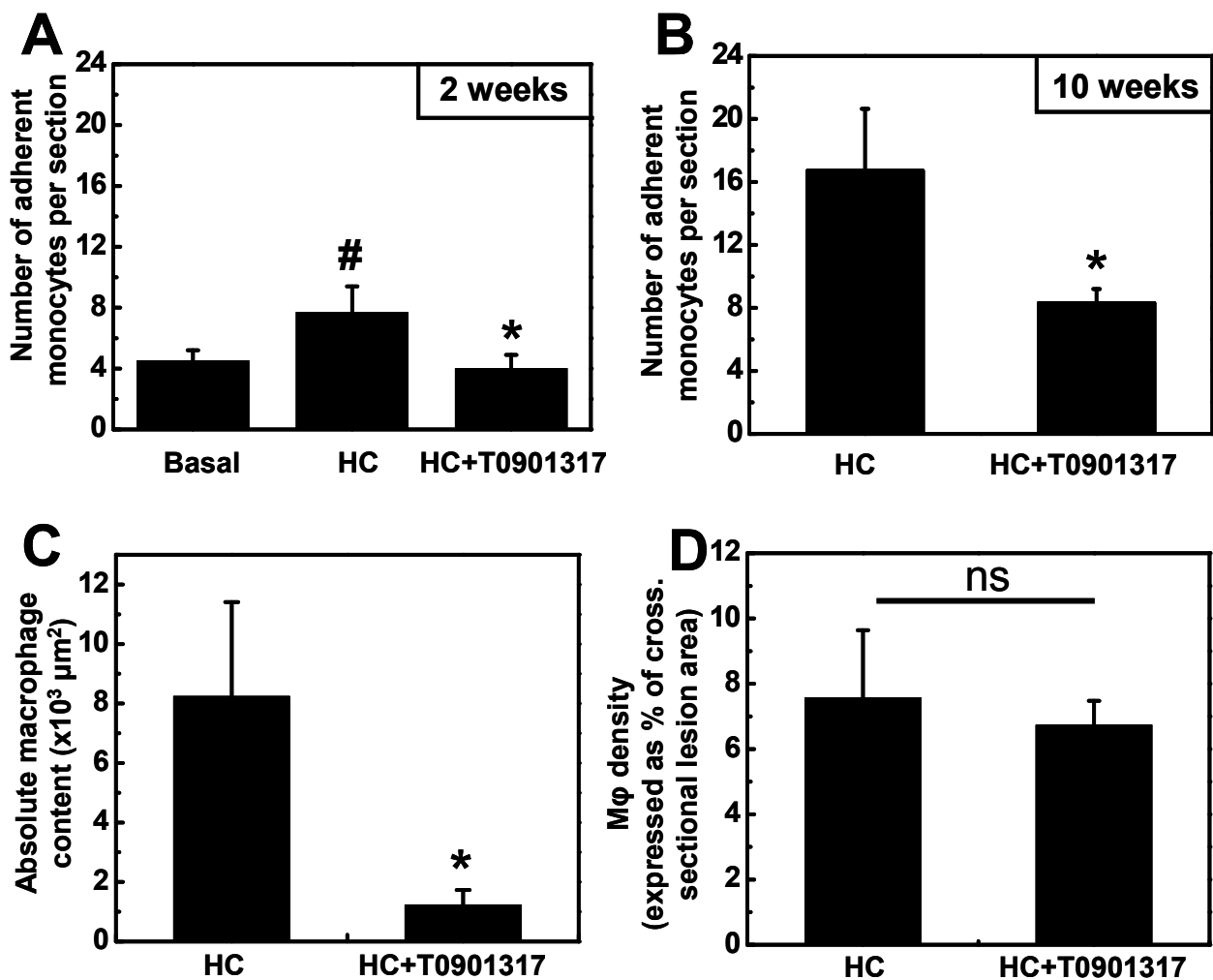


Figure 5. Effect of T0901317 on aspects of atherogenesis in the aortic valve area. Shown are effects of T0901317 on number of monocytes adhering to endothelium after 2 weeks (A) and 10 weeks of HC (B), on absolute macrophage containing lesion area after 10 weeks (C), and on macrophage density (i.e. macrophage area expressed as percentage of cross-sectional lesion area) after 10 weeks of HC. Data are presented as mean \pm SD, # $P < 0.05$ vs. chow-fed E3L mice (basal) and * $P < 0.05$ vs. HC.

The T0901317-reduced expression of adhesion molecules is paralleled by diminished recruitment of blood monocytes. As early as 2 weeks after starting HC-treatment, the

number of adherent monocytes was significantly increased in HC (7.7 ± 1.7 compared with 4.5 ± 0.7 in chow fed E3L mice (Figure 5A)), but not so in HC+T0901317 (4.0 ± 0.9). After 10 weeks, monocyte adhesion was further increased in HC (16.7 ± 4.0 ; Figure 5B); monocyte adherence was also elevated in HC+T0901317 (8.3 ± 2.1 vs. 4.0 ± 0.9 at $t=2$ weeks; $P < 0.05$), but remained significantly lower than in HC. Since monocyte-adhesion is a prerequisite, but not sufficient, for formation of early lesions, we subsequently determined lesional macrophage content and macrophage density. Figure 5C shows that the absolute macrophage-containing lesion area at 10 weeks was substantially (by 85%; $P < 0.05$) reduced in HC+T0901317 compared with HC ($1227 \pm 507 \mu\text{m}^2$ vs. $8238 \pm 3168 \mu\text{m}^2$). On the other hand, the macrophage density (the macrophage area expressed as percentage of the cross-sectional lesion area) is very similar in the two groups (Figure 5D).

Markedly, in HC+T0901317 lesions are less severe, with lesion progression seemingly blocked at the transition from lesion severity stage II to III (Fig. 2E), a phenotype that is also seen in CD44-deficient mice.¹⁹ CD44 is a cellular adhesion molecule that promotes adhesion and recruitment of monocytes/macrophages and regulates the migration and proliferation of SMC.^{19, 20} CD44 expression after 10 weeks of HC-treatment is mainly confined to monocytes and macrophage/foam cells in the intima, and expression is further increased and extended to SMC during subsequent atherogenesis progression after 15 weeks (Figure 6A-B). T0901317 strongly and significantly reduced CD44 expression in the vasculature (Figures 6A-C), both at the mRNA (RT-PCR analysis) and protein (immunohistochemical analysis) level. In contrast to CD44, T0901317 strongly increased the expression of genes reportedly involved in reverse cholesterol efflux (ApoE, ABCA1 and ABCG1; Table 3). Collectively, the data show that in HC+T0901317 lesion number, area, and severity are strongly suppressed with a parallel decrease in monocyte adhesion and SMC migration and proliferation. These effects can at least partly be explained by diminished expression of the adhesion molecules E-selectin, ICAM-1, and CD44, and by an up-regulation of factors promoting cholesterol efflux, ApoE, ABCA1 and ABCG1.

	HC	HC+ T0901317
<i>apoe</i>	100% \pm 11%	211% \pm 22.3% *
<i>abca1</i>	100% \pm 15%	821% \pm 211% *
<i>abcg1</i>	100% \pm 13%	491% \pm 109% *

Table 3. Vascular gene expression of control and T0901317-treated, cholesterol-fed E3L mice. Shown are the average aortic gene expression levels of *apoe*, *abca1*, and *abcg1* at the end of the 10-weeks treatment period as percentage values relative to corresponding values at the start ($t=0$) of the treatment (means \pm SD; $n=12$ for each group; * $P < 0.05$ vs. HC).

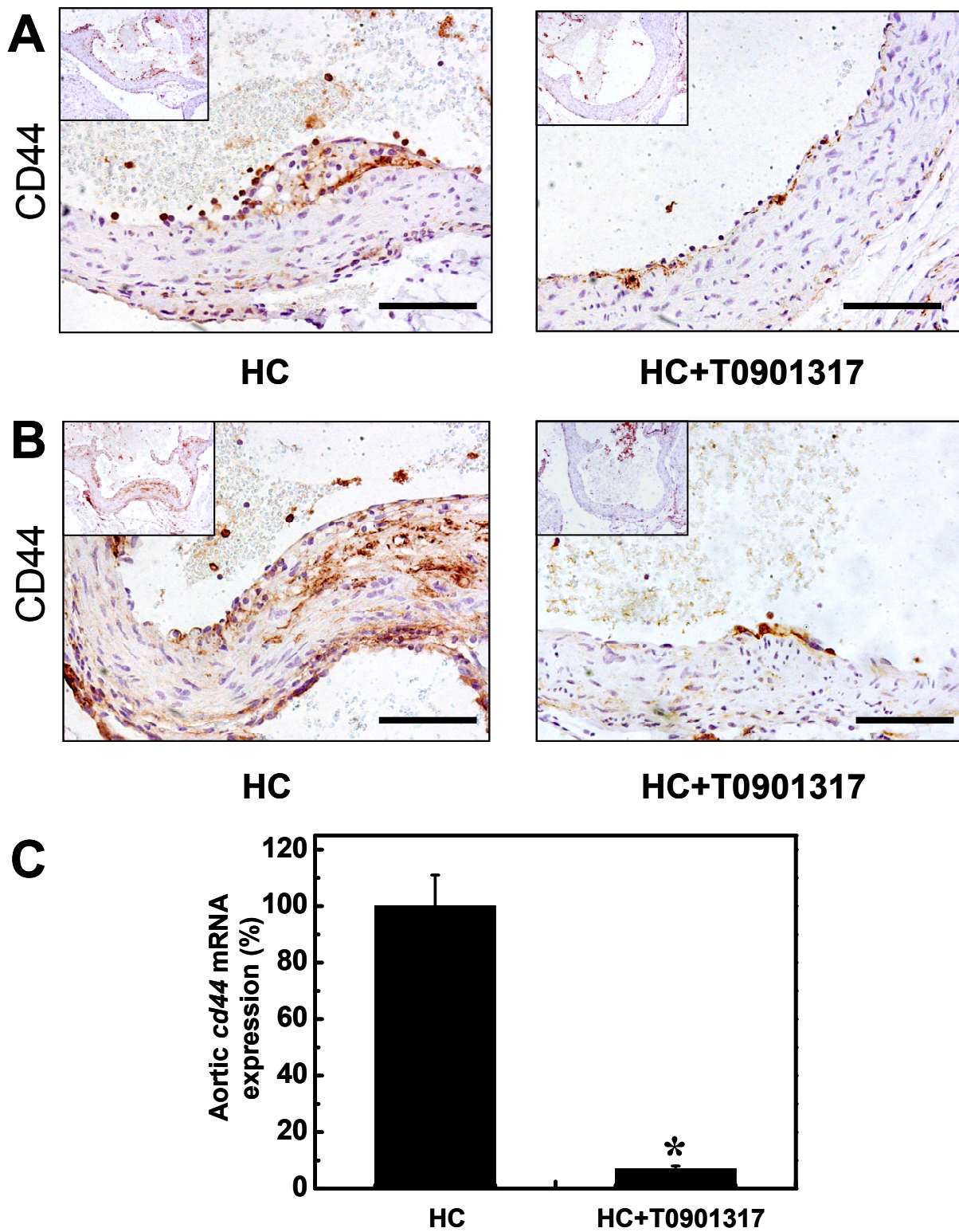


Figure 6. Effect of T0901317 on vascular CD44 expression. Representative photomicrographs of immunohistochemical staining of CD44-IR in cross-sections of the aortic root after treatment with HC or HC+T0901317 for 10 weeks (**A**) and 15 weeks (**B**). Bar= 100 μ m. Shown is also aortic *cd44* mRNA expression after 10 weeks of HC or HC+T0901317 (**C**). * $P < 0.05$ vs. HC.

Effect of T0901317 on pre-existing lesions

Considering the anti-inflammatory and anti-atherosclerotic activities of T0901317, the question arises whether T0901317 can also promote lesion regression. To address this question E3L mice were fed HC-diet for 18 weeks to induce moderately severe (type III-IV) lesions. One group of animals was then sacrificed (reference group) and two others received a cholesterol-free regression diet (RD) for 8 weeks, supplemented (RD+T0901317) or not (RD) with T0901317. The switch from HC to RD resulted in a fall in total plasma cholesterol from 21.2 ± 5.6 mM to 6.4 ± 2.3 mM ($P < 0.05$, Figure 7A) in the RD group, but no significant change in plasma cholesterol levels was seen in RD+T0901317 (20.7 ± 2.4 mM). Lipoprotein distribution profile analysis (Figure 7B) shows that the decrease in plasma cholesterol in RD is mainly in the VLDL range and to a lesser extent in the IDL/LDL range, but not in the HDL fractions, whereas the profile in RD+T0901317 very much resembles that seen in HC+T0901317, with an increase of cholesterol in fractions 10-17, corresponding to particles in the IDL/LDL range. Whereas lesion area in RD ($85924 \pm 31016 \mu\text{m}^2$) was not significantly decreased as compared to the reference group ($97203 \pm 16432 \mu\text{m}^2$), it was markedly reduced (by 76%) in RD+T0901317 ($20699 \pm 7995 \mu\text{m}^2$; $P < 0.05$; Figure 7C).

In both RD and RD+T0901317, a strong decrease in the plaque content of foam cells was observed: from $18040 \pm 3205 \mu\text{m}^2$ in the reference group to $2876 \pm 315 \mu\text{m}^2$ in RD ($P < 0.05$) and $363 \pm 351 \mu\text{m}^2$ in RD+T0901317 ($P < 0.05$; Figure 7D). Similarly, a significant drop ($P < 0.05$; Figure 7E) in monocyte adherence was seen in the two groups: from 23 ± 5 monocytes per section (reference) to 16 ± 4 (RD) and 10 ± 2 (RD+T0901317) monocytes per section.

A number of molecular mechanisms underlying regression in mice have been proposed, including macrophage apoptosis²¹, CCR7-mediated migration of foam cells to lymph nodes^{22, 23} and reverse cholesterol transport.²⁴ RT-PCR gene expression analysis (Table 4; values normalized for the number of macrophages and reference mice set at 100%) revealed strongly increased expression of the apoptotic genes BCL-2, Caspase-3, BAX, and FAS in aortas from RD mice, and even further enhanced in RD+T0901317 mice. The presence of condensed nuclei in the lesions (Figure 7F) illustrates the occurrence of ongoing apoptosis in RD+T0901317 mice.

Recently, Trogan et al.²² reported that the chemokine receptor CCR7 is expressed in foam cells and functionally required for regression. QRT-PCR analysis shows that CCR7 gene expression is significantly increased in RD ($225\% \pm 47\%$; $P < 0.05$) and even more so in

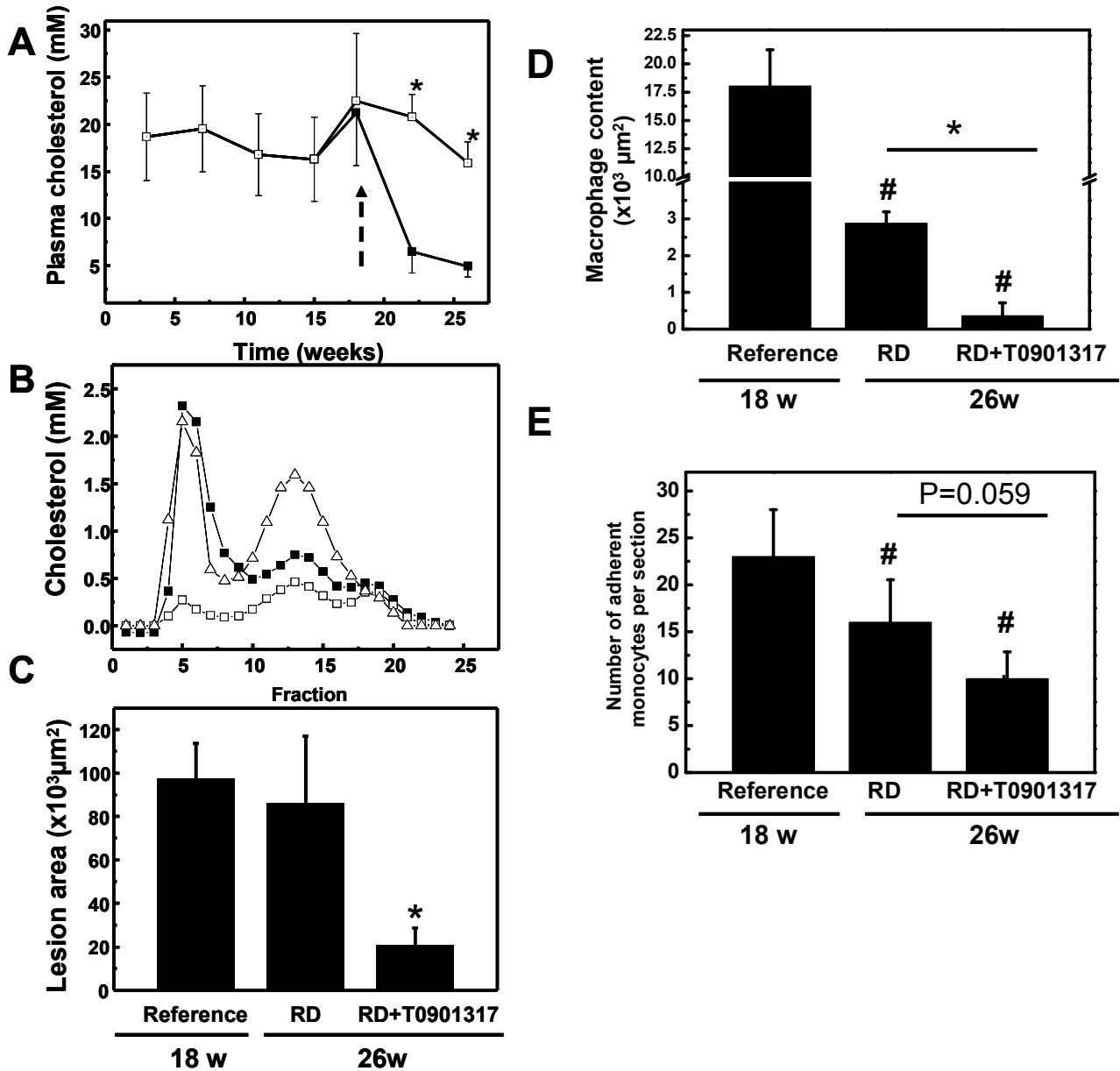


Figure 7: Effect of T0901317 on aspects of atherosclerotic lesion regression. Shown are **(A)** plasma cholesterol concentrations during regression period (arrow indicates start of RD feeding), with solid squares indicating RD treatment group and open squares indicating RD+T0901317 group. Data are represented as mean \pm SD, * P <0.05 vs. RD; **(B)** Lipoprotein distribution profiles of the two experimental groups ($n=8$ for each group) after 8 weeks of RD (open squares) or RD+T0901317 (solid squares). For comparison, the lipoprotein profile of pooled plasma of the reference group ($n=10$; $t=18$ weeks) is also shown (triangles); **(C)** total cross-sectional lesion area of reference, RD, and RD+T0901317 groups, with data representing mean values \pm SEM ($n=8$ per group); **(D)** absolute macrophage containing lesion area, with data representing mean values \pm SEM ($n=8$ mice per group); **(E)** number of monocytes adhering to endothelium, with data representing mean values \pm SEM ($n=8$ mice per group). **(Figure 7 is continued on next page)**

RD+T0901317 ($861\% \pm 132\%$; $P < 0.01$), indicating that CCR7 could also be a key factor in atherosclerosis regression in the present study. This is underlined by demonstrating enhanced CCR7-stained leukocytes accumulated in the adventitial space (Figure 7G).

Finally, we sought evidence supportive for increased reverse cholesterol efflux by measuring gene expression of cholesterol efflux pathway-related factors. QRT-PCR analysis of ApoE ($87\% \pm 10\%$), ABCA1 ($24\% \pm 3\%$; $P < 0.05$) and ABCG1 ($62\% \pm 6\%$; $P < 0.05$) showed decreased gene expression in RD as compared with the reference group, while T0901317 strongly induced these genes under regression conditions (ApoE: $306\% \pm 20\%$; ABCA1: $149\% \pm 24\%$ and ABCG1: $314\% \pm 29\%$; $P < 0.05$, Table 4), even if the macrophage content in RD+T0901317 was stronger decreased than that in RD.

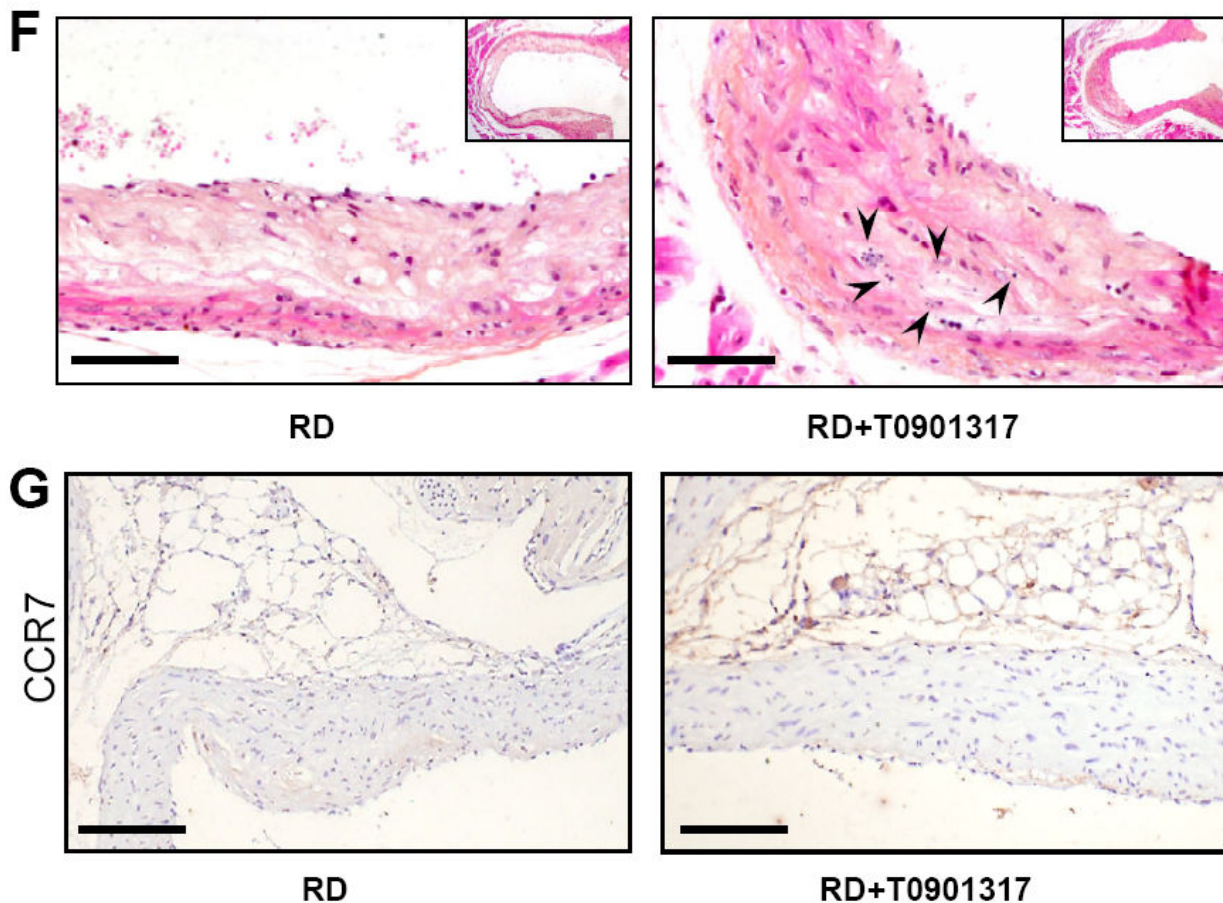


Figure 7: Effect of T0901317 on aspects of atherosclerotic lesion regression. (continued) (F) Representative photomicrographs illustrating condensed nuclei (arrowheads), (G) Representative photomicrographs of immunohistochemical staining of adventitial CCR7-IR.

Discussion

We evaluated the effects of an LXR agonist, T0901317 on specific aspects of atherosclerotic lesion progression and regression in E3L mice, a model for atherosclerosis with predictive value for the human situation. In E3L, vascular LXR α and LXR β expression was induced in aortic EC, SMC and macrophages under atherogenic dietary conditions. T0901317 strongly suppresses atherosclerotic lesion evolution and promotes lesion regression with respect to lesion number, area, and severity, despite elevated plasma total

cholesterol and triglyceride levels. T0901317 exerts its actions at several levels. Under lesion-*progressive* conditions T0901317 suppresses dietary cholesterol-induced vascular expression of the inflammatory transcription factor, NF- κ B, adhesion molecules (E-selectin, ICAM-1, and CD44), monocyte adhesion, and lesional macrophage content. T0901317 also induces the expression of the cholesterol efflux-related genes ApoE, ABCA1 and ABCG1, and blocks lesion evolution at the transition from stage II to III.

	t=18 weeks	Regression t=26 weeks	
	Reference	RD	RD+T0901317
<i>fas</i>	100% \pm 4%	291% \pm 11%*	2726% \pm 148%*,#
<i>bax</i>	100% \pm 6%	876% \pm 87%*	3072% \pm 374%*,#
<i>bcl-2</i>	100% \pm 6%	897% \pm 83%*	6162% \pm 376%*,#
<i>caspase-3</i>	100% \pm 5%	434% \pm 34%*	2173% \pm 386%*,#
<i>ccr7</i>	100% \pm 16%	225% \pm 47%*	861% \pm 132%*,#
<i>apoe</i>	100% \pm 11%	87% \pm 10%	306% \pm 20%*,#
<i>abca1</i>	100% \pm 8%	24% \pm 3%*	149% \pm 24%*,#
<i>abcg1</i>	100% \pm 7%	62% \pm 6%*	314% \pm 29%*,#

Table 4: Vascular gene expression of control and T0901317-treated E3L mice on a cholesterol-free, regressive diet (RD). Shown are average aortic gene expression levels of cholesterol-efflux-related (*apoe*, *abca1*, and *abcg1*) and apoptosis/regression-related genes (*fas*, *bax*, *bcl-2*, *caspase-3*, and *ccr7*) at the end of the regression period (t= 26 weeks) relative to reference group which was set at 100%. Values are normalized for aortic monocyte/macrophage content (means \pm SD; *P<0.05 vs. Reference and #P<0.05 vs. RD).

The ability of T0901317 to promote regression of preexisting atherosclerotic lesions under regressive conditions is linked mechanistically to i) increased cholesterol efflux from macrophages in the aortic lesions (through induction of expression of ApoE, ABCA1 and ABCG1); ii) reduction of macrophage content of the lesions by apoptosis; and iii) increased expression of the chemokine receptor CCR7, a factor functionally required for regression. The T0901317-induced increase in plasma cholesterol and triglycerides in E3L has also been described previously²⁵ and is similar to findings in ApoE-/- mice²⁶. The T0901317-induced rise in cholesterol in E3L is confined to particles co-eluting with the IDL/LDL fraction and very much resembles findings described for fenofibrate-treated E3L mice¹⁴.

The induced fraction contains mainly the apolipoprotein ApoE, to a minor extent ApoB, and no ApoA1. In contrast, the T0901317-induced increase in cholesterol in ApoE^{-/-} was in HDL and accompanied by a marked increase in ApoA1, but not ApoB levels²⁶. Interestingly, the apolipoprotein composition and size of the T0901317-induced particle in E3L is comparable to that induced by T0901317 in C57/Bl6 mice which was designated “enlarged HDL”²⁷. The particle also shows the characteristics of HDL1, which has been suggested to be involved in ApoA1-independent cholesterol efflux, possibly sustained by ApoE^{28, 29}.

Different effects of LXR agonists on cholesterol have been described in Ldlr^{-/-} mice. In one report, T0901317 did not affect plasma total cholesterol levels in Ldlr^{-/-} mice fed an atherogenic diet⁸, and in other studies a decrease in plasma cholesterol levels was observed with LXR agonists^{5, 6}.

A common finding in studies using T0901317 or other LXR ligands is the rise in triglyceride levels, which can be ascribed to the induction of the transcription factor SREBP-1 and its target genes FAS and SCD, two key enzymes involved in lipogenesis^{3, 4}. Noteworthy, T0901317 also is a high-affinity ligand for the xenobiotic receptor, pregnane-X-receptor (PXR). Because PXR and the LXRs are co-expressed in liver and PXR plays an important role in lipid metabolism, some of the hepatic effects of T0901317 may have resulted from simultaneous stimulation of LXR and PXR activation³⁰. Importantly, vascular effects of T0901317 reported here most likely reflect LXR activation only since CD36, a PXR target³¹ was not induced by T0901317 in E3L mice (data not shown).

Our finding that T0901317 strongly suppresses atherosclerotic lesion evolution in E3L mice is in line with previous gain-of-function and loss-of-function studies⁷ and results obtained in ApoE^{-/-} and Ldlr^{-/-} mice with T0901317 and the LXR agonist GW3965^{5, 8}. Relevantly, the present study reveals several levels of anti-atherosclerotic action of T0901317. Firstly, a strong reduction of aortic inflammation as reflected by diminished vascular endothelial NF-κB activity, adhesion molecule expression (E-selectin, ICAM-1, and CD44), monocyte adhesion, and lesional macrophage content, all in the absence of systemic inflammation (cf. SAA). Secondly, T0901317 strongly increases gene expression of factors important for cholesterol efflux from vascular macrophages (ApoE, ABCA1, and ABCG1). ABCA1 mediates cholesterol efflux from macrophages to lipid-free ApoA1³² whereas ABCG1 is a mediator of macrophage cholesterol efflux to mature HDL *in vitro*³³. Up-regulation of ABCA1 and ABCG1 in the vasculature has previously been suggested to contribute to the anti-atherogenic capacity of T0901317^{5, 8}. Another LXR agonist, GW3965 was shown to increase aortic expression of ABCA1 and ABCG1 in ApoE^{-/-} mice⁵.

Thirdly, T0901317 blocked lesion progression from lesion severity stage II to III, characterized by the absence of SMC in the cap¹⁵. Proliferation and migration of SMC from the media towards the intima, a hallmark of type III lesions, is a key event in lesion progression and regulated by CD44^{34, 35}. The detected down-regulation of CD44 by T0901317 in our study may thus provide an explanation for the observed blockage of lesion progression.

Atherosclerosis regression is an important clinical goal, but mouse studies on regression are relatively few. We observed that under dietary cholesterol-free (“regressive”) conditions, there was a rapid loss of plaque foam cells from existing plaques, and this process was further enhanced by T0901317. In line with a previous study in E3L mice macrophage disappearance preceded reduction in plaque size under regressive conditions¹¹. Remarkably, the effect of T0901317 on plaque regression occurred while plasma cholesterol level and lipoprotein profile were very similar to those found with T0901317 under progressive (HC) conditions. Also, monocyte adherence in the presence of T0901317 was not different between regressive and progressive conditions.

Three processes were identified that could be relevant for explaining the T0901317-stimulated disappearance of macrophages. First, the disappearance of macrophages could be the result of apoptosis. Indeed, we found evidence for increased number of apoptotic bodies with a parallel up-regulation of pro-apoptotic genes (Caspase-3, BAX, and FAS) in the aorta. Secondly, T0901317 enhances gene expression of reverse cholesterol transport markers, ApoE, ABCA1 and ABCG1. This is of relevance because it is thought that the elevated plasma membrane cholesterol content in foam cells inhibits cell migration³⁶. Since plaque progression may result in part from long-term retention of foam cells in the plaque, promoting emigration of foam cells from atherosclerotic lesions through lowering cellular cholesterol content might lead to plaque regression. Thirdly, findings from a study by Trogan *et al.*²² raised the possibility that, during regression, foam cells acquire dendritic cell (DC) characteristics that permit them to migrate to draining lymph nodes. This migration depends on the chemokine receptor CCR7, which might become up-regulated in foam cells in plaques undergoing regression. When CCR7 function was abrogated *in vivo* by treatment with antibodies to CCR7 ligands CCL19 and CCL21, lesion size and foam cell content were substantially preserved. In line with this, we found that CCR7 expression in the vasculature is located at the adventitial space and is increased 2-fold when regression is induced by RD and 8-fold by RD+T0901317, despite

the decreases in macrophage content. Concomitantly, increases in hepatic CCL19 and CCL21 expression were observed (data not shown).

In summary, our findings show a comprehensive analysis of the beneficial effects of T0901317 on lesion evolution and regression in E3L mice and provide plausible mechanisms for its mode of action, including stimulation of cholesterol efflux-related genes and suppression of the inflammatory state. The data also revealed CCR7 induction in foam cells, a chemokine receptor reportedly functionally required for regression. LXR agonists could thus contribute in abrogating (pre-existing) atherosclerosis.

Acknowledgements

This study was supported by the Dutch Heart Foundation (NHS; grant 2002B102 to L.V.), the Center for Medical Systems Biology CMSB (grant 115 to J.d.V.v.d.W), and the Dutch Organization for Scientific Research (NWO; grant VENI 016. 036.061 to R.K.).

Reference List

- (1) Braunwald E. Shattuck lecture--cardiovascular medicine at the turn of the millennium: triumphs, concerns, and opportunities. *N Engl J Med* 1997 November 6;337(19):1360-9.
- (2) Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. *Circulation* 2002 March 5;105(9):1135-43.
- (3) Schultz JR, Tu H, Luk A, Repa JJ, Medina JC, Li L, Schwendner S, Wang S, Thoolen M, Mangelsdorf DJ, Lustig KD, Shan B. Role of LXRs in control of lipogenesis. *Genes Dev* 2000 November 15;14(22):2831-8.
- (4) Zelcer N, Tontonoz P. Liver X receptors as integrators of metabolic and inflammatory signaling. *J Clin Invest* 2006 March;116(3):607-14.
- (5) Joseph SB, McKilligin E, Pei L, Watson MA, Collins AR, Laffitte BA, Chen M, Noh G, Goodman J, Hagger GN, Tran J, Tippin TK, Wang X, Lusis AJ, Hsueh WA, Law RE, Collins JL, Willson TM, Tontonoz P. Synthetic LXR ligand inhibits the development of atherosclerosis in mice. *Proc Natl Acad Sci U S A* 2002 May 28;99(11):7604-9.
- (6) Levin N, Bischoff ED, Daige CL, Thomas D, Vu CT, Heyman RA, Tangirala RK, Schulman IG. Macrophage liver X receptor is required for antiatherogenic activity of LXR agonists. *Arterioscler Thromb Vasc Biol* 2005 January;25(1):135-42.
- (7) Tangirala RK, Bischoff ED, Joseph SB, Wagner BL, Walczak R, Laffitte BA, Daige CL, Thomas D, Heyman RA, Mangelsdorf DJ, Wang X, Lusis AJ, Tontonoz P, Schulman IG. Identification of macrophage liver X receptors as inhibitors of atherosclerosis. *Proc Natl Acad Sci U S A* 2002 September 3;99(18):11896-901.
- (8) Terasaka N, Hiroshima A, Koieyama T, Ubukata N, Morikawa Y, Nakai D, Inaba T. T-0901317, a synthetic liver X receptor ligand, inhibits development of atherosclerosis in LDL receptor-deficient mice. *FEBS Lett* 2003 February 11;536(1-3):6-11.
- (9) Zadelaar S, Kleemann R, Verschuren L, de Vries-van der Weij, van der HJ, Princen HM, Kooistra T. Mouse models for atherosclerosis and pharmaceutical modifiers. *Arterioscler Thromb Vasc Biol* 2007 August;27(8):1706-21.

- (10) Kleemann R, Verschuren L, van Erk MJ, Nikolsky Y, Cnubben NH, Verheij ER, Smilde AK, Hendriks HF, Zadelaar S, Smith GJ, Kaznacheev V, Nikolskaya T, Melnikov A, Hurt-Camejo E, van der GJ, van OB, Kooistra T. Atherosclerosis and liver inflammation induced by increased dietary cholesterol intake: a combined transcriptomics and metabolomics analysis. *Genome Biol* 2007 September 24;8(9):R200.
- (11) Gijbels MJ, van der CM, van der Laan LJ, Emeis JJ, Havekes LM, Hofker MH, Kraal G. Progression and regression of atherosclerosis in APOE3-Leiden transgenic mice: an immunohistochemical study. *Atherosclerosis* 1999 March;143(1):15-25.
- (12) Verschuren L, Kleemann R, Offerman EH, Szalai AJ, Emeis SJ, Princen HM, Kooistra T. Effect of low dose atorvastatin versus diet-induced cholesterol lowering on atherosclerotic lesion progression and inflammation in apolipoprotein E*3-Leiden transgenic mice. *Arterioscler Thromb Vasc Biol* 2005 January;25(1):161-7.
- (13) Rein D, Schijlen E, Kooistra T, Herbers K, Verschuren L, Hall R, Sonnewald U, Bovy A, Kleemann R. Transgenic flavonoid tomato intake reduces C-reactive protein in human C-reactive protein transgenic mice more than wild-type tomato. *J Nutr* 2006 September;136(9):2331-7.
- (14) Kooistra T, Verschuren L, de Vries-van der Weij, Koenig W, Toet K, Princen HM, Kleemann R. Fenofibrate reduces atherogenesis in ApoE*3Leiden mice: evidence for multiple antiatherogenic effects besides lowering plasma cholesterol. *Arterioscler Thromb Vasc Biol* 2006 October;26(10):2322-30.
- (15) Stary HC. Natural history and histological classification of atherosclerotic lesions: an update. *Arterioscler Thromb Vasc Biol* 2000 May;20(5):1177-8.
- (16) Kleemann R, Princen HM, Emeis JJ, Jukema JW, Fontijn RD, Horrevoets AJ, Kooistra T, Havekes LM. Rosuvastatin reduces atherosclerosis development beyond and independent of its plasma cholesterol-lowering effect in APOE*3-Leiden transgenic mice: evidence for antiinflammatory effects of rosuvastatin. *Circulation* 2003 September 16;108(11):1368-74.
- (17) Bernhagen J, Krohn R, Lue H, Gregory JL, Zerneck A, Koenen RR, Dewor M, Georgiev I, Schober A, Leng L, Kooistra T, Fingerle-Rowson G, Ghezzi P, Kleemann R, McColl SR, Bucala R, Hickey MJ, Weber C. MIF is a noncognate ligand of CXC chemokine receptors in inflammatory and atherogenic cell recruitment. *Nat Med* 2007 May;13(5):587-96.
- (18) Verschuren L, Lindeman JH, Bockel JH, Abdul-Hussien H, Kooistra T, Kleemann R. Up-Regulation and Coexpression of MIF and Matrix Metalloproteinases in Human Abdominal Aortic Aneurysms. *Antioxid Redox Signal* 2005 September;7(9-10):1195-202.
- (19) Zhao L, Hall JA, Levenkova N, Lee E, Middleton MK, Zukas AM, Rader DJ, Rux JJ, Pure E. CD44 regulates vascular gene expression in a proatherogenic environment. *Arterioscler Thromb Vasc Biol* 2007 April;27(4):886-92.
- (20) Cuff CA, Kothapalli D, Azonobi I, Chun S, Zhang Y, Belkin R, Yeh C, Secreto A, Assoian RK, Rader DJ, Pure E. The adhesion receptor CD44 promotes atherosclerosis by mediating inflammatory cell recruitment and vascular cell activation. *J Clin Invest* 2001 October;108(7):1031-40.
- (21) Wang BY, Ho HK, Lin PS, Schwarzacher SP, Pollman MJ, Gibbons GH, Tsao PS, Cooke JP. Regression of atherosclerosis: role of nitric oxide and apoptosis. *Circulation* 1999 March 9;99(9):1236-41.
- (22) Trogan E, Feig JE, Dogan S, Rothblat GH, Angeli V, Tacke F, Randolph GJ, Fisher EA. Gene expression changes in foam cells and the role of chemokine receptor CCR7 during atherosclerosis regression in ApoE-deficient mice. *Proc Natl Acad Sci U S A* 2006 March 7;103(10):3781-6.
- (23) Llodra J, Angeli V, Liu J, Trogan E, Fisher EA, Randolph GJ. Emigration of monocyte-derived cells from atherosclerotic lesions characterizes regressive, but not progressive, plaques. *Proc Natl Acad Sci U S A* 2004 August 10;101(32):11779-84.

- (24) Cuchel M, Rader DJ. Macrophage reverse cholesterol transport: key to the regression of atherosclerosis. *Circulation* 2006 May 30;113(21):2548-55.
- (25) Grefhorst A, Elzinga BM, Voshol PJ, Plosch T, Kok T, Bloks VW, van der Sluijs FH, Havekes LM, Romijn JA, Verkade HJ, Kuipers F. Stimulation of lipogenesis by pharmacological activation of the liver X receptor leads to production of large, triglyceride-rich very low density lipoprotein particles. *J Biol Chem* 2002 September 13;277(37):34182-90.
- (26) Dai X, Ou X, Hao X, Cao D, Tang Y, Hu Y, Li X, Tang C. Effect of T0901317 on Hepatic Proinflammatory Gene Expression in ApoE^{-/-} Mice Fed a High-fat/high-cholesterol Diet. *Inflammation* 2007 August;30(3-4):105-17.
- (27) Jiang XC, Beyer TP, Li Z, Liu J, Quan W, Schmidt RJ, Zhang Y, Bensch WR, Eacho PI, Cao G. Enlargement of high density lipoprotein in mice via liver X receptor activation requires apolipoprotein E and is abolished by cholesteryl ester transfer protein expression. *J Biol Chem* 2003 December 5;278(49):49072-8.
- (28) Gruen ML, Plummer MR, Zhang W, Posey KA, Linton MF, Fazio S, Hasty AH. Persistence of high density lipoprotein particles in obese mice lacking apolipoprotein A-I. *J Lipid Res* 2005 September;46(9):2007-14.
- (29) Vaisman BL, Lambert G, Amar M, Joyce C, Ito T, Shamburek RD, Cain WJ, Fruchart-Najib J, Neufeld ED, Remaley AT, Brewer HB, Jr., Santamarina-Fojo S. ABCA1 overexpression leads to hyperalphalipoproteinemia and increased biliary cholesterol excretion in transgenic mice. *J Clin Invest* 2001 July;108(2):303-9.
- (30) Mitro N, Vargas L, Romeo R, Koder A, Saez E. T0901317 is a potent PXR ligand: implications for the biology ascribed to LXR. *FEBS Lett* 2007 May 1;581(9):1721-6.
- (31) Zhou J, Zhai Y, Mu Y, Gong H, Uppal H, Toma D, Ren S, Evans RM, Xie W. A novel pregnane X receptor-mediated and sterol regulatory element-binding protein-independent lipogenic pathway. *J Biol Chem* 2006 May 26;281(21):15013-20.
- (32) Wang N, Silver DL, Costet P, Tall AR. Specific binding of ApoA-I, enhanced cholesterol efflux, and altered plasma membrane morphology in cells expressing ABC1. *J Biol Chem* 2000 October 20;275(42):33053-8.
- (33) Wang N, Lan D, Chen W, Matsuura F, Tall AR. ATP-binding cassette transporters G1 and G4 mediate cellular cholesterol efflux to high-density lipoproteins. *Proc Natl Acad Sci U S A* 2004 June 29;101(26):9774-9.
- (34) Lazaar AL, Albelda SM, Pilewski JM, Brennan B, Pure E, Panettieri RA, Jr. T lymphocytes adhere to airway smooth muscle cells via integrins and CD44 and induce smooth muscle cell DNA synthesis. *J Exp Med* 1994 September 1;180(3):807-16.
- (35) Jain M, He Q, Lee WS, Kashiki S, Foster LC, Tsai JC, Lee ME, Haber E. Role of CD44 in the reaction of vascular smooth muscle cells to arterial wall injury. *J Clin Invest* 1996 August 1;98(3):877.
- (36) Nagao T, Qin C, Grosheva I, Maxfield FR, Pierini LM. Elevated cholesterol levels in the plasma membranes of macrophages inhibit migration by disrupting RhoA regulation. *Arterioscler Thromb Vasc Biol* 2007 July;27(7):1596-602.

