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Macrophage Independent Regulation of Reverse Cholesterol Transport by Liver X Receptors

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Abstract

Objective—The ability of high density lipoprotein (HDL) particles to accept cholesterol from peripheral cells such as lipid-laden macrophages and to transport cholesterol to the liver for catabolism and excretion in a process termed reverse cholesterol transport (RCT) is believed to underlie the beneficial cardiovascular effects of elevated HDL. The liver X receptors (LXR α and LXR β) regulate RCT by controlling the efflux of cholesterol from macrophages to HDL and the excretion, catabolism and absorption of cholesterol in the liver and intestine. Importantly, treatment with LXR agonists increases RCT and decreases atherosclerosis in animal models. Nevertheless, LXRs are expressed in multiple tissues involved in RCT and their tissue specific contributions to RCT are still not well defined.

Approach and Results—Utilizing tissue-specific LXR deletions together with *in vitro* and *in vivo* assays of cholesterol efflux and fecal cholesterol excretion we demonstrate that macrophage LXR activity is neither necessary nor sufficient for LXR agonist-stimulated RCT. In contrast, the ability of LXR agonists primarily acting in the intestine to increase HDL mass and HDL function appears to underlie the ability of LXR agonists to stimulate RCT *in vivo*.

Conclusions—We demonstrate that activation of LXR in macrophages makes little or no contribution to LXR agonist-stimulated RCT. Unexpectedly our studies suggest that the ability of macrophages to efflux cholesterol to HDL *in vivo* is not regulated by macrophage activity but is primarily determined by the quantity and functional activity of HDL.

Keywords

Reverse cholesterol transport; Liver X receptors; Macrophage; HDL; Atherosclerosis

INTRODUCTION

Cardiovascular disease (CVD) is a leading cause of death globally and it is well established that elevated levels of cholesterol in the blood is a major contributor to disease development¹. Excess plasma cholesterol accumulates in macrophages lodged in blood vessel walls which along with an associated inflammatory response initiate the formation of

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DISCLOSURES

The authors have nothing to disclose.

atherosclerotic lesions². Statin therapy is highly effective for lowering disease-causing low-density lipoprotein (LDL) cholesterol thereby reducing morbidity and mortality associated with CVD³. Nevertheless, the residual risk for major cardiac events remains high for patients receiving LDL lowering therapies prompting the search for complementary therapeutic approaches⁴. Epidemiological studies have demonstrated that levels of high density lipoprotein particle (HDL) cholesterol are inversely associated with CVD suggesting the potential therapeutic benefit of raising HDL⁵. Recent clinical trials with cholesteryl ester transfer protein (CETP) inhibitors and niacin, however, have failed to demonstrate clinical benefits of increasing HDL cholesterol^{6, 7}. The clinical trial results have led to the suggestion that HDL functionality, rather than the absolute mass of HDL cholesterol may be a more accurate indicator for CVD risk^{8, 9}. The ability of HDL to promote cholesterol efflux from macrophage foam cells within atherosclerotic lesions was one of its earliest recognized functions^{10, 11}. Importantly, cholesterol efflux from foam cells has been shown to increase macrophage egression and to reduce lesion burden in animal models of cardiovascular disease^{12–14}. Measuring the dynamic rate of macrophage cholesterol efflux, therefore, may be a better predictor of the anti-atherogenic effects of novel HDL-targeted therapies¹⁵.

The movement of cholesterol from peripheral cells such as macrophages to HDL constitutes the first step in a process termed reverse cholesterol transport (RCT). HDL-derived cholesterol is then trafficked to the liver where it is catabolized or excreted to the bile^{16, 17}. Recent studies have also described hepatic-independent pathways for cholesterol secretion¹⁸. Studies in animal models indicate that measurements of RCT can strongly predict the effect of genetic and pharmacological manipulations on atherosclerosis¹⁹. Similarly, in humans an inverse relationship has been uncovered between the ability of patient sera to accept cholesterol from macrophages *in vitro* and measurements of carotid intima media thickness with cholesterol acceptor capacity being a strong predictor of coronary disease status¹⁵. The utility of *in vitro* measurements of plasma cholesterol acceptor activity for predicting CVD as well as the proteins/particles in human sera responsible for accepting cholesterol, however, remain controversial^{20, 21}.

Integral to the regulation of RCT are the liver X receptors, LXR α (NR1H3) and LXR β (NR1H2), which are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors. Studies using genetic knockouts and synthetic agonists have defined important roles for LXRs in the control of cholesterol homeostasis and fatty acid metabolism^{22–24}. Treatment of animals with LXR agonists results in changes in gene expression promoting the efflux of cholesterol from peripheral cells such as macrophages, the secretion of cholesterol from the liver, and the inhibition of cholesterol absorption in the intestine²². Importantly, the endogenous ligands for LXRs are oxidized forms of cholesterol (oxysterols) that increase coordinately with intracellular cholesterol levels, thus allowing these receptors to act as sensors to maintain appropriate cholesterol levels throughout the body^{25, 26}. At the molecular level, LXRs control macrophage cholesterol efflux by regulating expression of genes encoding the ATP-binding cassette (ABC) transporters ABCA1 and ABCG1 as well the gene encoding apolipoprotein E (APOE)²². Up-regulation of ABCA1 and ABCG1 results in increased transfer of intracellular cholesterol to HDL particles, and genome-wide association studies have linked both transporters to HDL

cholesterol levels in humans^{27, 28}. Mutations in the human ABCA1 gene results in a genetic syndrome referred to as Tangier disease. Tangier disease patients characteristically present with little or no HDL, massive accumulation of cholesterol in lymph tissues and are at increased risk for atherosclerosis^{19, 29, 30}. LXR also regulates expression of ABCG5 and ABCG8, two half-transporters that dimerize to form an additional cholesterol transporter^{31, 32}. Expression of ABCG5/ABCG8 is largely restricted to the liver and intestine, where these proteins function to promote the excretion of cholesterol (liver) and limit cholesterol absorption (intestine)³³. Genetic deletion of ABCG5/G8 or deletion of LXR α in the liver largely blocks the ability of LXR agonists to stimulate fecal excretion of cholesterol^{34, 35}. Thus activation of LXRs promotes a net movement of cholesterol from the periphery out of the body. Not surprisingly, LXR agonists decrease atherosclerosis in animal models of CVD^{34, 36–38}.

Treatment with LXR agonists also increases plasma HDL cholesterol^{34, 39} suggesting LXRs can regulate RCT in both a cell autonomous fashion, by controlling the transporters required to mobilize intracellular cholesterol, as well as in a non-autonomous fashion by regulating the amount of cholesterol acceptor in plasma. Interestingly, the ability of LXR agonists to increase HDL cholesterol levels is largely mediated by the induction of ABCA1 expression in the intestine^{34, 40}. Not unexpected then is the observation that an intestinal-specific LXR agonist increases RCT⁴¹. Although LXR agonists appear to act in macrophages, the liver and the intestines to stimulate RCT, studies utilizing genetic knockouts indicate that macrophages are the major site of LXR agonist-dependent anti-atherogenic activity^{38, 42, 43}. The atherosclerosis studies therefore led us to question the tissue-specific contributions of LXRs to the regulation of RCT. Combining *in vivo* measurements with tissue-selective knockouts we show that the ability of LXRs to regulate HDL quantity and activity is a major driver of RCT. In contrast, macrophage LXR activity is neither necessary nor sufficient. Furthermore, our studies suggest that the ability of macrophages to efflux cholesterol to HDL *in vivo* is primarily determined by the quantity and functional activity of HDL in the surrounding environment.

MATERIALS AND METHODS

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

RESULTS

Macrophage LXR is not necessary for LXR agonist-dependent RCT

LXR activity in the liver and the macrophage is thought to contribute to RCT⁴⁴ but the relative contribution of LXR at these sites has not been well defined. To determine the contribution of macrophage LXR to RCT, we injected bone marrow derived macrophages (BMM) that had been loaded with ³H-cholesterol *in vitro* into the peritoneal space of mice and followed the movement of macrophage-derived cholesterol to the plasma and ultimately to the feces as described by Naik et al.⁴⁵. For these studies we used C57BL/6J (LXR⁺) and *Lxr α ^{-/-}/Lxr β ^{-/-}* (DKO) mice in the C57BL/6J background to generate three groups of animals: LXR⁺ macrophage introduced into LXR⁺ mice (referred to as Mac^{LXR⁺}/LXR⁺), LXR⁺ macrophage introduced into DKO mice (referred to as Mac^{LXR⁺}/DKO) and DKO

macrophages into LXR⁺ mice (referred to as Mac^{DKO}/LXR⁺). For the RCT experiments age-matched male mice were treated with vehicle or the LXR agonist T0901317 (10mpk) daily by oral gavage for 3 days prior to injection. Following injection of radiolabeled macrophage, mice continued to be treated with vehicle or agonist for the duration of the experiment (for a total of 5 doses) and the appearance of ³H sterol was quantitated in the plasma at 6, 24 and 48 hours after injection. At completion of the experiment (48 hours) the amount of ³H-sterol in the feces and liver was determined. In preliminary experiments we found that LXR activation (e.g. rise in plasma triglycerides) can be observed following 3 doses of T0901317 at 10mpk and that the plasma concentrations of T0901317 are similar between C57BL/6J and *Lxrα*^{-/-}/*Lxrβ*^{-/-} mice and at least 10 times above the reported EC₅₀ (data not shown).

As expected, agonist treatment of Mac^{LXR+}/LXR⁺ mice stimulates the appearance of macrophage-derived cholesterol in plasma over the time course and in the feces at 48 hours (Figure 1A–B). When LXR is present only in macrophages (Mac^{LXR+}/DKO), however, the amount of macrophage-derived cholesterol in the plasma and feces is significantly decreased (Figure 1A–B). Similarly, the ability of T0901317 to increase the accumulation of macrophage-derived cholesterol in the plasma of Mac^{LXR+}/DKO mice is decreased by 70% (Figure 1A) and agonist-stimulated fecal excretion is completely blocked in these animals (Figure 1B). Quantification of ABCA1 mRNA levels in macrophage re-extracted from the peritoneal space at completion of the experiment demonstrates that placing LXR⁺ macrophages into DKO mice does not impair macrophage LXR transcriptional activity (Figure 1C). In contrast to the decreased RCT observed in the Mac^{LXR+}/DKO mice, selective deletion of LXR in macrophages (Mac^{DKO}/LXR⁺) has little or no effect on either the accumulation of ³H-cholesterol in the plasma or the feces (Figure 1A–B). Little or no differences among the groups are seen when hepatic levels of ³H-sterols were examined (Supplemental Figure I). To further address the contribution of macrophage LXR activity to the ability of LXR agonists to increase the accumulation of macrophage-derived cholesterol in the plasma we examined ³H-cholesterol levels in vehicle and T0901317 treated Mac^{LXR+}/LXR⁺ and Mac^{DKO}/LXR⁺ mice at 30, 60 and 90 minutes after introducing radiolabeled macrophage into the peritoneal space. As shown in Figure 1D, pretreatment of mice with T0901317 significantly increases ³H-cholesterol in the plasma by 60 minutes. Even at these short time points, however, the LXR genotype of the macrophages has no effect on the response to agonist treatment. The observation that LXR macrophage activity does not appear to play a role in the accumulation of ³H-cholesterol in the plasma *in vivo* is consistent with studies *in vitro* demonstrating that ABCA1 expression and cholesterol efflux is actually slightly increased in *Lxrα*^{-/-}/*Lxrβ*^{-/-} macrophages⁴⁶. In the absence of agonists LXRs repress transcription by interacting with corepressors and this activity is lost upon genetic deletion⁴⁶. A similar up-regulation of ABCA1 expression is observed in DKO macrophages recovered from the peritoneal space of LXR⁺ mice after *in vivo* RCT experiments (Figure 1C).

HDL levels and adipose activity drive LXR-agonist-dependent RCT

LXR agonists are known to increase HDL cholesterol predominately by increasing expression of ABCA1 in the intestine⁴⁰. Consistent with an LXR agonist-dependent increase

in HDL cholesterol (Table 1), plasma from T0901317 treated C57BL/6J (LXR+) mice has increased cholesterol acceptor activity *in vitro* when ^3H -cholesterol loaded RAW264.7 cells are used as donor macrophages. The effect of agonist, however, is lost when plasma from DKO animals is used (Figure 2A). To further address the contribution of HDL to macrophage efflux, a similar series of *in vitro* efflux experiments were carried out using FPLC-purified HDL particles (Figure 2B). For experiments with FPLC-purified HDL, peak HDL fractions were pooled (Supplemental Figure II) and normalized by the amount of apolipoprotein AI (APOAI) as determined by Western blotting (Supplemental Figure IIIA). Using APOAI as a relative measure for particle number, HDL from agonist treated C57BL/6J accept greater amounts of macrophage cholesterol compared to DKO mice (Figure 2B). Together these experiments show that LXR agonist treatment increases both HDL mass and HDL function.

Over the course of *in vivo* RCT experiments it is likely that macrophage-derived ^3H -cholesterol incorporates into cells and tissues throughout the body. Thus along with increasing the cholesterol acceptor activity of HDL, LXR agonists may also increase the amount of cholesterol in plasma by promoting efflux from other tissues via transcriptional up-regulation of ABCA1, ABCG1 and APOE. To address the possible contributions of different tissues to LXR agonist-stimulated RCT, radiolabeled LXR+ macrophages were introduced into vehicle and T0901317 treated LXR+ mice ($\text{Mac}^{\text{LXR+}}/\text{LXR+}$) and multiple tissues were harvested at 48 hours post injection to determine if agonist treatment promotes a net loss in tissue-associated ^3H -sterols. As shown in Figure 2C, a significant agonist-dependent decrease is observed in white adipose (gonadal fat pad) suggesting that fat tissue may make an important contribution to LXR-stimulated accumulation of cholesterol in the plasma and feces. T0901317-dependent changes in ^3H -sterol levels were not observed in other tissues (Figure 2C). Importantly, the decrease in adipose ^3H -sterol levels could result from increased LXR transcriptional activity in fat cells, the improved acceptor activity of HDL or both.

Diet-dependent regulation of Liver LXR activity and RCT

We have previously determined under severe hyperlipidemic conditions ($Ldlr^{-/-}$ mice on Western diet) that liver-specific deletion of LXR α impairs the accumulation of macrophage-derived cholesterol in both the plasma and in the feces³⁴. To further investigate the contribution of liver LXR activity to RCT, liver-specific knockout LXR α (LivKO) mice³⁴ and floxed littermate controls (carrying the floxed LXR α allele without albumin CRE) were placed on a standard chow diet with or without 0.2% cholesterol. LXR α is the major LXR subtype expressed in the liver⁴⁷ and the ability of T0901317 to increase plasma triglycerides and to induce expression of hepatic ABCG5, ABCG8 and ABCA1 is significantly impaired in LivKO mice³⁴ (Table 1 and Supplemental Figure IV). After 4 weeks on diet, plasma total cholesterol increases 30–50% in both LivKO and littermate control groups fed the 0.2% cholesterol diet (Table 1). Consistent with published data, the 0.2% cholesterol diet also significantly increases hepatic cholesterol in LivKO mice due to impaired fecal excretion and decreased bile acid synthesis^{34, 47} (Supplemental Figure VA). Hepatic triglycerides, however, are not increased (Supplemental Figure VB) and the increase in hepatic cholesterol measured in LivKO mice does not result in a significant increase in liver damage

(Supplemental Figure VC–D), markers of inflammation or markers of endoplasmic reticulum stress (data not shown). For the final week of the diet treatment (week 4) mice were treated with vehicle or T0901317 and RCT was measured *in vivo* as in previous experiments by introducing radiolabeled LXR+ macrophages. On a standard chow diet the appearance of ³H-cholesterol in the plasma of T0901317 treated LivKO and littermate controls is significantly increased at 24 and 48 hours (Figure 3A) indicating that liver LXRα activity is not required for agonists to increase the accumulation of ³H-cholesterol in the plasma. On the other hand, the ability of LXR agonists to increase fecal sterol excretion is completely lost in LivKO mice (Figure 3B) a result consistent with decreased agonist-dependent regulation of ABCG5 and ABCG8 in the livers of these animals (Supplemental Figure IV). Interestingly, exposure to the 0.2% cholesterol diet impairs both LXR agonist-dependent plasma and fecal cholesterol accumulation in LivKO mice relative to controls (Figure 3C–D). Thus dietary cholesterol uncovers a critical role for hepatic LXR activity in controlling the accumulation of macrophage-derived cholesterol in plasma. The ability of LXR agonists to increase HDL cholesterol levels in LivKO mice is also sensitive to dietary cholesterol (Figure 4A and Table 1) despite similar increases in the intestinal mRNA levels of ABCA1 (Supplemental Figure VI). Furthermore a dietary cholesterol-dependent decrease in cholesterol acceptor activity is also observed when FPLC-purified HDL particles isolated from T0901317 treated LivKO mice are compared to HDL particles from littermate controls *in vitro* (Figure 4B; see Supplemental Figures II and IIIC–D for FPLC profiles and APOA1 levels). The reason(s) why the cholesterol enriched diet impairs the ability of LXR agonist treatment to increase HDL mass and function remains to be determined. Nevertheless, the failure of T0901317 to modulate HDL levels and functional activity in cholesterol fed LivKO mice supports the hypothesis that the ability of LXR agonists to promote the accumulation of macrophage-derived cholesterol in plasma is largely derived from systemic effects on HDL and independent of macrophage LXR activity.

Our results indicate that LXR activation can improve the cholesterol acceptor activity of HDL and this effect is influenced by liver LXR activity in a diet-dependent fashion. As an initial characterization of HDL particle composition we measured phospholipid levels in the FPLC-purified HDL fractions. Phospholipids are the major components by mass of HDL and a number of studies suggest that HDL phospholipid levels are a better predictor of cholesterol efflux than other HDL parameters^{48, 49}. As shown in Figure 4C and 4D, T0901317 treatment increases the amount of total phospholipids associated with purified HDL particles (normalized by APOA1 levels) from standard chow fed floxed and LivKO mice (Figure 4C). The increase in HDL-phospholipid levels is consistent with studies demonstrating that LXR agonist treatment increased HDL particle size^{34, 50}. The effect of agonist treatment on HDL-phospholipid levels, however, is lost in 0.2% cholesterol diet challenged LivKO animals (Figure 4D). Phospholipid transfer protein is a HDL-bound protein that plays a major role in regulating HDL size and phospholipid composition through its phospholipid transfer activity⁵¹. Phospholipid transfer protein mRNA levels have been shown to be regulated by LXR⁵² however we did not detect significant differences in plasma phospholipid transfer protein activity between floxed and LivKO mice on either dietary condition (Supplemental Table I).

CETP decreases macrophage-derived cholesterol in plasma

To test the hypothesis that LXR-dependent regulation of HDL levels and activity plays a major role in driving the accumulation of macrophage-derived cholesterol in plasma, we took advantage of the observation that LXR agonist-dependent increases in HDL cholesterol are lost in CETP transgenic mice⁵³. CETP facilitates the transfer of cholesterol esters from HDL to apolipoprotein B containing particles thereby decreasing HDL cholesterol levels⁵⁴. Importantly, the transgene is under control of the human CETP promoter which has been shown to be directly regulated by LXR in human cells and in transgenic mice^{55, 56} (Supplemental Figure VIIA–B). Indeed, treatment of CETP transgenic mice with T0901317 decreases HDL cholesterol by approximately 25% and raises the amount of cholesterol associated with apolipoprotein B containing lipoprotein particles (Figure 5A and B and Table 1). To determine the effect of CETP expression on RCT *in vivo*, CETP transgenic mice and littermate controls were treated with vehicle or T0901317 and injected with ³H-cholesterol loaded C57BL/6J (LXR⁺) BMM as described in previous experiments. Consistent with a critical role for HDL in promoting the accumulation of macrophage-derived cholesterol in plasma, the amount of ³H-cholesterol in this compartment at 24 and 48 hours is significantly reduced in CETP transgenic mice and the ability of T0901317 to increase plasma cholesterol accumulation is lost (Figure 5C). Similarly, unfractionated plasma and FPLC purified HDL particles from T0901317 treated CETP transgenic mice do not exhibit increased efflux activity as is observed in non-transgenic controls (Figure 5D–E). The ability of LXR agonists to increase HDL phospholipids, however, is not impaired in CETP transgenics (Supplemental Figure VIIC). Taken together, the RCT and *in vitro* efflux experiments indicate that LXR-dependent up-regulation of CETP expression counters the ability of agonists to enhance the appearance of macrophage-derived cholesterol in the plasma. In contrast to the inhibitory effect of CETP expression on the accumulation of macrophage-derived cholesterol in plasma, LXR agonist treatment increases fecal ³H-sterol levels in both CETP transgenic and littermate controls (Figure 5F). Interestingly, CETP expression also results in a significant increase in fecal bile acids in vehicle treated animals (Supplemental Figure VIID). Increased bile acid synthesis has previously been reported in CETP transgenic mice^{57, 58}. Little or no difference was observed in hepatic ³H-cholesterol levels among the groups (data not shown). Thus as observed with the LXR α liver-specific knockout mice (LivKO), it is possible to functionally sever the transfer of macrophage-derived cholesterol to the plasma from subsequent fecal excretion.

DISCUSSION

The discovery that LXR agonists can promote macrophage cholesterol efflux *in vitro* via direct regulation of the genes encoding ABCA1, ABCG1 and APOE^{22, 59} suggested a simple hypothesis for the cardio-protective effect of LXR activation based on promoting cholesterol transfer from macrophage foam cells to HDL; the first step in the RCT pathway. This hypothesis is supported by the finding that macrophage LXR activity is required for the anti-atherogenic activity of LXR agonists³⁸. Combining *in vitro* cholesterol efflux measurements, *in vivo* RCT assays and tissue-specific LXR knockouts we now demonstrate that the ability of LXR agonists to stimulate RCT *in vivo* defined as the transfer of macrophage-derived cholesterol to the feces is largely independent of macrophage LXR

activity (Figure 6). Thus macrophage LXRs are neither necessary nor sufficient for LXR agonists to increase RCT, at least when measured in an acute assay over a 48 hour time course. Additionally, our studies suggest that it is the ability of LXR agonists to increase HDL biogenesis and to improve HDL functional activity that is largely responsible for stimulating the appearance of macrophage-derived cholesterol in plasma (Figure 6). The LXR agonist used in these studies, T0901317, has been reported to modulate other nuclear receptors, at least *in vitro*^{60–62}. Therefore the possibility that another nuclear receptor, such as the pregnane X receptor, contributes to the activity of this molecule *in vivo* cannot be ruled out. All the activities of T0901317 measured in this work, however, are lost in cells and animals that are deficient in LXRs.

On a standard mouse chow diet the ability of LXR agonists to stimulate the accumulation of macrophage-derived cholesterol in plasma is independent of LXR activity in both macrophages and the liver. Previous studies have determined that LXR agonists increase HDL cholesterol by inducing ABCA1 expression in the intestine^{34, 40, 63}. Consistent with an important role for intestinal LXR activity in regulating RCT is the finding that selective activation of LXRs in the intestine using either a poorly absorbed “intestine-specific” LXR agonist⁴¹ or intestine-specific transgenic over expression of a hyperactive LXR (VP16-LXR α)⁶⁴ increases RCT when measured using assays similar to those described in this work. Furthermore, our studies indicate that intestinal LXR activation can increase the cholesterol acceptor activity of HDL particles (Figure 6) most likely by increasing the production of immature nascent particles that have been shown to be preferred cholesterol acceptors^{65–67}. Interestingly, this work also describes a potential role for LXR activity in white adipose in regulating cholesterol trafficking.

To test the hypothesis that agonist dependent increases in HDL mass and function drive the accumulation of macrophage-derived cholesterol in plasma during RCT assays we took advantage of the observation that the ability of LXR agonists to raise HDL cholesterol is lost in CETP transgenic mice^{53, 56}. CETP, an enzyme that transfers cholesterol esters from HDL to apolipoprotein B containing lipoprotein particles in exchange for triglycerides, is not expressed in rodents but the human gene used in this study is regulated by LXRs^{55, 56, 68}. Importantly CETP activity in the plasma is increased following LXR agonist treatment, HDL levels are lowered and plasma cholesterol accumulation measured during RCT assays is decreased. The cholesterol acceptor activity of unfractionated plasma and FPLC-purified HDL from T0901317 treated CETP transgenic mice is also reduced relative to non-transgenic controls. Finally, the conclusion that increasing CETP activity impairs HDL particle function is consistent with reports that inhibition of CETP activity improves the cholesterol acceptor activity of human HDL particles⁶⁹. Taken together, the data supports the hypothesis that the ability of LXR agonists to increase the accumulation of macrophage-derived cholesterol in plasma is primarily determined by the quantity and quality of the HDL particles. Nevertheless, in CETP transgenic animals LXR agonist treatment still increases fecal excretion of macrophage-derived cholesterol. Therefore we cannot rule out the possibility that CETP expression decreases the levels of macrophage-derived cholesterol in plasma by increasing hepatic clearance via receptors for apolipoprotein B containing particles. Similar to CETP expression, Bi et al. found that liver-specific deletion of ABCA1

reduces plasma HDL levels and decreases plasma accumulation of ^3H -cholesterol in RCT assays without altering fecal sterol excretion⁶³. Bi et al. suggest the small plasma HDL pool that remains in the liver ABCA1 knockout mice may be quantitatively sufficient to mediate the transport of macrophage-derived cholesterol to the liver for excretion⁶³. Our study with CETP transgenic mice together with the work of Bi et al. raises the possibility, at least under these experimental conditions, that the appearance of macrophage-derived cholesterol in the plasma is a not a rate limiting step for fecal cholesterol excretion.

In contrast to CETP transgenic expression, liver-specific deletion of LXR α (LivKO) has little or no effect on the accumulation of macrophage-derived cholesterol in plasma (on a standard chow diet) but strongly inhibits LXR agonist-stimulated fecal cholesterol excretion (Figure 6). Thus our analysis of CETP transgenic and LXR α LivKO mice indicate that it is possible to functionally separate plasma cholesterol accumulation from fecal excretion. Plasma cholesterol accumulation is primarily controlled by the ability of LXRs to regulate the quantity and quality of HDL while fecal excretion is controlled by LXR-dependent regulation of hepatic ABCG5 and ABCG8 levels allowing a single transcription factor pair (LXR α and LXR β) to coordinate cholesterol movement throughout the body. These results raise the question regarding the potential therapeutic benefit of regulating either macrophage cholesterol efflux or fecal excretion independently. Current therapeutic approaches for atherosclerotic cardiovascular disease all involve reducing low density lipoprotein (LDL) cholesterol in the blood. Therefore if increasing fecal cholesterol excretion ultimately reduces plasma LDL levels one might predict a therapeutic benefit. On the other hand, APOA Milano and other APOA1-derived peptides have been shown to increase macrophage cholesterol efflux and to improve cardiovascular endpoints, although it not clear that the beneficial effects of these agents are dependent on promoting cholesterol efflux^{70, 71}. Future studies that for instance combine macrophage selective over expression of ABCA1 with LXR liver-specific knockouts may be a way to address the therapeutic benefits of increased macrophage efflux in the absence of fecal cholesterol excretion.

Interestingly, the contribution of liver LXR activity to RCT can be influenced by the cholesterol content of the diet. As described above, on a standard mouse chow diet knocking out LXR α in the liver has little or no effect on the accumulation of macrophage-derived cholesterol in plasma while completely eliminating agonist-stimulated fecal excretion (Figure 6). When cholesterol (0.2%) is added to the diet, however, LXR agonist-dependent plasma cholesterol accumulation is significantly decreased in LivKO mice. The absence of agonist-dependent accumulation of macrophage-derived cholesterol in plasma when cholesterol is included in the diet correlates with the inability of agonist treatment to increase HDL cholesterol and to improve the acceptor capacity of purified HDL in LivKO mice under these conditions. LXR agonist treatment still increases ABCA1 expression in the intestines of LivKO on the 0.2% cholesterol diet and the reason(s) why HDL cholesterol levels are not increased in these mice remains to be determined. Compared to littermate floxed controls on the 0.2% cholesterol diet, LivKO mice have increased hepatic cholesterol levels although we did not detect any evidence for increased hepatic inflammation, endoplasmic reticulum stress or liver damage in these mice. We and others have shown that the ability of LXR agonists to increase HDL levels in LXR positive animals is lost under

severe hyperlipidemic conditions such as *Ldlr*^{-/-} or *ApoE*^{-/-} mice on Western diets^{34, 36, 37, 39, 72}. Thus the ability of LXR agonists to regulate HDL metabolism can be influenced by dietary cholesterol levels. Interestingly, Kalaany et al. demonstrated that *Lxrα*^{-/-}/*Lxrβ*^{-/-} mice are resistant to high fat diet-induced obesity, however, this resistance is only observed when the high fat diet also contains cholesterol⁷³. These observations raise the possibility that hepatic cholesterol accumulation leads to the generation of a paracrine signal that can influence lipid metabolism in other tissues.

Bone marrow transplantation experiments and over expression studies indicate that macrophages are the site of LXR agonist-dependent anti-atherogenic activity^{38, 42, 43}. The studies described in this work, however, indicate that macrophage LXR activity does not make a significant contribution to RCT. Similarly using LivKO mice in a severe hyperlipidemic environment (*Ldlr*^{-/-} + Western diet) we demonstrated that LXR agonists can reduce atherosclerosis without increasing RCT³⁴. Kappus et al. also reached an analogous conclusion in a recent study using mice with myeloid-specific double knockout of *Abca1* and *Abcg1*⁷⁴. Together, these observations suggests that while hematopoietic LXR expression is required for the beneficial effects of LXR agonists an increase in RCT or macrophage efflux is not. LXR activation inhibits NFκβ signaling suggesting decreased inflammation as an obvious mechanism for LXR-dependent anti-atherogenic activity^{75, 76}. A dominant role for anti-inflammatory activity as the beneficial effect of LXR activation on atherosclerosis has important implications for the potential therapeutic use of LXR agonists. In particular, *in vitro* experiments have suggested that LXR agonists can have pro-inflammatory activities in human macrophages⁷⁷ in contrast to the anti-inflammatory effects measured in rodents. Additionally, as described above, pre-clinical studies examining the anti-atherogenic activity of LXR ligands generally have been carried out under severe hyperlipidemic conditions where the ability of LXR agonists to increase HDL mass is lost^{34, 37, 78}. Since human cardiovascular disease patients do not usually present with the supra-physiological plasma cholesterol levels observed in genetic mouse models, the ability of LXR agonists to stimulate RCT may be maintained in humans and could be therapeutic. As we observe in CETP transgenic mice, however, the ability of LXR agonists to increase HDL cholesterol appears to be lost in non-human primates that express CETP^{79, 80}.

Recent clinical trials with niacin⁷ and CETP inhibitors⁶ have called into question the hypothesis that raising HDL cholesterol has beneficial effects on human cardiovascular disease. The clinical trials together with experiments suggesting that the cholesterol acceptor activity of HDL isolated from patients can be a more accurate measurement of cardiovascular disease risk has led to the proposal that assessing HDL function may be more relevant than measurements of HDL cholesterol mass^{9, 15, 20}. Along with increasing the levels of HDL cholesterol, LXR agonist treatment also increases the cholesterol acceptor activity of HDL particles that were normalized by the quantity of APOA1. HDL particles are heterogeneous in size and composition making it difficult to discern the LXR-dependent modifications that improve cholesterol acceptor activity. Nevertheless, our initial analysis of HDL particle composition found increased levels of phospholipids (normalized to APOA1) in the HDL particles purified from agonist treated animals. The phospholipid:APOA1 ratio in HDL has been shown to be an important determining factor in predicting macrophage

efflux. Studies using mice and rats expressing human APOA1 indicate that the prime component of HDL that modulates cholesterol efflux is HDL phospholipid^{81, 82}. Furthermore, the correlation between macrophage cholesterol efflux and HDL phospholipid in human sera is stronger than with any other measured lipoprotein parameter, including HDL cholesterol, APOA1 and triglycerides⁴⁸. CETP expression, however, appears to impact HDL function without modulating phospholipid levels suggesting that multiple components of HDL can influence particle function. LXRs likely regulate multiple pathways that modulate HDL activity and future studies employing detailed lipidomic and proteomic approaches can be used to further define the LXR-dependent changes in HDL composition that regulate HDL particle function. These studies that define particle function may open the door to new therapeutic approaches for targeting HDL.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

ABCA1	ATB binding cassette transporter A1
ABCG1	ATB binding cassette transporter G1
ABCG5	ATB binding cassette transporter G5
ABCG8	ATB binding cassette transporter G8
APOA1	apolipoprotein A1
CETP	cholesteryl ester transfer protein
CVD	cardiovascular disease
FPLC	fast liquid protein chromatography
HDL	high density lipoprotein
LDL	low density lipoprotein
LXR	liver X receptor
RCT	reverse cholesterol transport

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SIGNIFICANCE

The liver X receptors, LXR α and LXR β , are important regulators of cholesterol transport. Treatment with LXR agonists promotes the efflux of cholesterol from macrophages and the excretion of cholesterol from the liver resulting in a net movement of cholesterol from the periphery out of the body. Utilizing tissue-specific LXR deletions we demonstrate that macrophage LXR activity is neither necessary nor sufficient for LXR agonist stimulated RCT. In contrast the ability of LXR agonists to increase HDL mass and HDL function primarily acting in the intestine appears to underlie the ability of LXR agonists to stimulate RCT *in vivo*.

Our studies suggest that the ability of macrophages to efflux cholesterol to HDL *in vivo* is not regulated in a cell autonomous fashion but is primarily determined by the quantity and functional activity of HDL.

Figure 1. Macrophage LXRs are not required for RCT

^3H -cholesterol and acetylated LDL-loaded C57BL6/J or DKO BMMs were injected into C57BL6/J or DKO mice to generate $\text{Mac}^{\text{LXR}+}/\text{LXR}+$, $\text{Mac}^{\text{LXR}+}/\text{DKO}$, and $\text{Mac}^{\text{DKO}}/\text{LXR}+$ mice (see text). Animals were treated for 3 days with or without 10 mg/kg T0901317 ($n=6/\text{group}$), and the amount of ^3H sterol in plasma (A and D) and feces (B) were determined as described in the Materials and Methods. Mice continued to receive vehicle or T0901317 treatment for the duration of the experiment. C) Total RNA was isolated from BMM that were recovered from the peritoneal space and the mRNA levels of *Abca1* were measured by quantitative real-time PCR. Data are mean $\pm$ SEM. *Statistically significant difference between vehicle and T0901317-treated animals of the same genotype ($p < 0.05$). † Statistically significant difference between $\text{Mac}^{\text{LXR}+}/\text{LXR}+$ and $\text{Mac}^{\text{LXR}+}/\text{DKO}$ or between $\text{Mac}^{\text{LXR}+}/\text{LXR}+$ and $\text{Mac}^{\text{DKO}}/\text{LXR}+$ mice with the same treatment ($p < 0.05$).

Figure 2. HDL function and adipose tissue drives LXR-dependent RCT

C57BL6/J and DKO mice (n = 5–6/group) were treated for 5 days with vehicle or 10 mg/kg T0901317 and *In vitro* macrophage cholesterol efflux was measured as described in Materials and Methods using ³H-cholesterol labeled Raw264.7 cells that were incubated with 0.03% pooled plasma (**A**) or FPLC purified HDL (**B**). Efflux data are representative of 3 independent experiments. **C**) ³H-cholesterol and acetylated LDL-loaded C57BL6/J BMM were injected into C57BL6/J mice treated for 3 days with or without 10 mg/kg T0901317 (n=6/group), and the amount of ³H sterol in tissues were determined as described in the Materials and Methods. Mice continued to receive vehicle or T0901317 treatment for the duration of the experiment. All data are expressed as mean ± SEM. *Statistically significant difference between vehicle and T0901317-treated animals of the same genotype (*p* 0.05%). † Statistically significant difference between C57BL6/J and DKO with the same treatment (*p* 0.05%).

Figure 3. *In vivo* RCT in chow and 0.2% cholesterol diet fed LivKO mice

^3H -cholesterol and acetylated LDL-loaded C57BL/6/J BMMs were injected into Floxed or LivKO mice fed standard chow (A and B) or 0.2% cholesterol supplemented diet (C and D). Animals were treated for 3 days with or without 10 mg/kg T0901317 ($n=6/\text{group}$) prior to BMM injection, and the amount of ^3H sterol in plasma (A and C) and feces (B and D) was determined as described in the Materials and Methods. Mice continued to receive vehicle or T0901317 treatment for the duration of the experiment. Data are mean $\pm$ SEM. *Statistically significant difference between vehicle and T0901317-treated animals of the same genotype ($p < 0.05$). † Statistically significant difference between Floxed and LivKO mice with the same treatment ($p < 0.05$).

Figure 4. LXR agonist dependent changes in HDL mass and function in LivKO mice

A) Plasma HDL cholesterol levels in chow and 0.2% cholesterol diet fed Floxed and LivKO mice ($n = 6/\text{group}$) treated for 5 days with vehicle or 10 mg/kg T0901317. **B)** *In vitro* macrophage cholesterol efflux was measured as described in Materials and Methods using ^3H -cholesterol labeled Raw264.7 cells that were incubated with FPLC purified HDL from animals treated with vehicle or T0901317 (10 mpk) for 5 days ($n=5-6/\text{group}$). Efflux data are representative of 3 independent experiments. Total phospholipids in FPLC purified HDL from Floxed and LivKO mice fed a standard chow diet (**C**) or 0.2% cholesterol diet (**D**) and treated for 5 days with vehicle or T0901317 (10 mpk). HDL phospholipid levels were normalized by HDL APOA1 protein levels as determined by Western blotting. Data are mean $\pm$ SEM. *Statistically significant difference between vehicle and T0901317-treated animals of the same genotype ($p < 0.05$). †Statistically significant difference between Floxed and LivKO mice with the same treatment and diet ($p < 0.05$).

Figure 5. CETP inhibits LXR agonist-dependent RCT

CETP^{-/-} (A) and CETP^{+/+} (B) mice (n=6/group) were treated with vehicle or T0901317 (10 mpk) for 5 days, plasma was pooled, subjected to FPLC and the cholesterol content of each fraction was measured as described in Materials and Methods. ³H-cholesterol and acetylated LDL-loaded C67BL/6 BMDMs were injected into CETP^{-/-} and CETP^{+/+} mice (n=6/group) treated with vehicle or T0901317 and the amount of ³H sterol in plasma (C) and feces (F) was determined as described in Materials and Methods. (D) Raw 264.7 cells were incubated with 0.03% pooled plasma (D) or FPLC purified HDL (E) from vehicle or T0901317 treated CETP^{-/-} and CETP^{+/+} mice (n=5/group) and cholesterol efflux was measured as described in Materials and Methods. Efflux data is representative of 3 independent experiments. Data are mean ± SEM. *Statistically significant difference between vehicle and T0901317-treated animals of the same genotype ($p < 0.05$). †Statistically significant difference between CETP^{-/-} and CETP^{+/+} with the same treatment ($p < 0.05$).

Figure 6. Effect of LXR agonist treatment on in vivo RCT

LXR agonist activity in the intestine and adipose tissue contributes to the accumulation of macrophage-derived cholesterol in the plasma during *in vivo* RCT assays. Agonist activity in the liver promotes fecal cholesterol excretion (see text for details).

Table 1

Plasma Lipid Levels.

Diet	Strain	Drug treatment	Total Cholesterol (mg/dL)	HDL- Cholesterol (mg/dL)	Triglycerides (mg/dL)
Chow	C57bl6/J	Vehicle	122.2 ± 5.4	65.9 ± 1.2	55.3 ± 3.6
		T0901317	155.4 ± 3.9*	100.0 ± 4.8*	90.5 ± 7.2*
Chow	<i>Lxrα^{-/-} Lxrβ^{-/-}</i>	Vehicle	113.6 ± 3.9	46.1 ± 1.7***	40.2 ± 2.7
		T0901317	112.5 ± 3.6	54.5 ± 1.8**	55.5 ± 5.2
Chow	Floxed	Vehicle	109.0 ± 8.1	65.3 ± 3.6	64.2 ± 7.8
		T0901317	163.1 ± 8.3*	121.5 ± 10.9*	113.4 ± 10.5*
Chow	LivKO	Vehicle	115.2 ± 9.4	44.9 ± 5.2**	45.1 ± 3.9
		T0901317	166.4 ± 9.9*	86.4 ± 6.7*	47.9 ± 3.1
0.2% cholesterol	Floxed	Vehicle	159.6 ± 12.5 [†]	73.9 ± 5.3	59.9 ± 3.4
		T0901317	216.9 ± 16.0** [†]	93.8 ± 5.4* [†]	197.2 ± 17.6* [†]
0.2% cholesterol	LivKO	Vehicle	166.7 ± 11.0 [†]	54.1 ± 2.5**	47.7 ± 7.2
		T0901317	167.9 ± 6.2	56.0 ± 5.6	35.4 ± 3.2
Chow	CETP-	Vehicle	116.9 ± 2.6	68.2 ± 3.3	52.6 ± 3.4
		T0901317	193.6 ± 6.8*	92.4 ± 1.7*	85.2 ± 7.5*
Chow	CETP+	Vehicle	105.3 ± 4.6	52.4 ± .9	57.0 ± 6.2
		T0901317	78.4 ± 4.6*	38.0 ± 1.9*	92.2 ± 9.2*

* Statistically significant difference between vehicle and T0901317-treated animals of the same genotype (n = 6, p = 0.05%).

[†] Statistically significant difference between chow and 0.2% cholesterol diet fed mice of the same genotype and treatment (n = 6, p = 0.05%).** Statistically significant difference between *C57bl6/J* and *Lxrα^{-/-} Lxrβ^{-/-}* or floxed and LivKO mice with the same treatment. Data are mean ± SEM.