The Role of the Liver X Receptors in Reverse Cholesterol Transport and Atherosclerosis

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A dissertation presented to the Graduate Faculty of the University of Virginia in Candidacy for the Degree of Doctor of Philosophy

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August 2014

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ABSTRACT

Liver X receptors (LXRα and LXRβ) are important regulators of cholesterol and lipid metabolism, and their activation has been shown to inhibit cardiovascular disease and reduce atherosclerosis in animal models. Believed to be integral to their anti-atherogenic effects, LXRs regulate reverse cholesterol transport (RCT), the process by which high density lipoprotein (HDL) particles transport cholesterol from peripheral cells such as lipid-laden macrophages to the liver for catabolism and excretion. LXRs regulate RCT by controlling cholesterol efflux from macrophages to HDL and the excretion, catabolism and absorption of cholesterol in the liver and intestine. Deletion of LXR activity in macrophages increases atherosclerosis; a consequence thought to result from the loss of LXR stimulated RCT. Nevertheless, the relative contribution of LXR activity in the macrophage, liver and intestine to LXR stimulated RCT has not been determined. Utilizing tissue-specific LXR deletions we demonstrate that macrophage LXR activity is neither necessary nor sufficient for LXR agonists to promote RCT. Furthermore, 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 quality of the HDL particles.

While macrophage LXR is not necessary for LXR agonist stimulated RCT, liver LXRα is required for agonist stimulated fecal cholesterol excretion. Interestingly, when the liver specific LXRα deficient mice are challenged with dietary cholesterol the ability of LXR agonists to increase macrophage efflux in is lost. To investigate if the loss of LXR agonist stimulated RCT increases atherosclerosis, we crossed the liver-specific LXRα deficient mouse into a pro-atherogenic background. Hyperlipidemic liver-specific

LXRα knockout animals had a significant reduction in agonist-stimulated macrophage cholesterol efflux and fecal cholesterol excretion, highlighting an important role for hepatic LXRα in regulating RCT and cholesterol metabolism. Deletion of liver LXRα also results in increased atherosclerosis, uncovering an important function for hepatic LXRα activity in limiting cardiovascular disease. Nevertheless, synthetic LXR agonists were still protective against atherosclerosis in the absence of hepatic LXRα. Together our data suggests that LXR agonist-stimulated RCT may not be necessary for the anti-atherogenic activity of LXR agonists; however, LXR expression in the macrophage is required for their protective effects. In addition to promoting cholesterol efflux LXR agonists exert anti-inflammatory effects in macrophages, and this function may in fact underlie their athero-protective activities. Future studies from this work might elucidate the contribution of macrophage LXR anti-inflammatory activity to the cardio-protective effects of LXR agonists. Such findings could provide valuable insight for future therapeutic strategies for the treatment of cardiovascular disease.

Acknowledgements

It is with tremendous respect and appreciation that I extend my gratitude to my mentor, Ira Schulman, for his excellent mentorship. Without his expertise, support and guidance none of this work would have been possible and the many invaluable skills that he has imparted to me will serve me well on my path to becoming a clinician-scientist. I would also like to thank the members of my doctoral committee for their time, interest and insightful questions and comments. It was a great pleasure sharing the last four years with many wonderful individuals in the Schulman lab and the Pharmacology Department. Their support, companionship and guidance made my PhD training quite an enjoyable experience. Lastly, I'm indebted to the individuals who inspired me to pursue science and medicine and who have continued to remain good friends and now colleagues: Dr. Michael Petelle, Dr. Wes McCoy, and Dr.Walter K. Schmidt. May I inspire and guide the next generation of clinicians/scientists as they so humbly did for me. Lastly, I'd like to thank my family, both related and otherwise, for their continuing to support, love and belief in me over the years.

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INFγ – interferon γ

HMG-CoA – 3-hydroxy-3-methylglutaryl coenzyme A

ER- endoplasmic reticulum

SREBP- sterol regulatory element-binding protein

LDL – low density lipoprotein

TAG – triacylglycerol

ApoE – apolioprotein E

ABCG5 – ATP-binding cassette sub-family G member 5

ABCG8 – ATP-binding cassette sub-family G member 8

CVD – cardiovascular disease

CHD – coronary heart disease

HDL – high density lipoprotein

PPARα – peroxisome proliferator-activated receptor α

SRA – scavenger receptor A

oxLDL – oxidized low-density lipoprotein

LDLR – low-density lipoprotein receptor

TLR – toll-like receptor

MyD88 – myeloid differentiation primary response 88

HSP60 – heat shock protein 60

ApoB – apolioprotein B

NMR – nuclear magnetic resonance

LDL-P – low-density lipoprotein particle number

CETP – cholesteryl ester transfer protein

LPL – lipoprotein lipase

MTP – microsomal triglyceride transfer protein

CV – cardiovascular

NFκB – nuclear factor κB

TNFα – tumor necrosis factor α

IL-6 – interleukin-6

IL-1β – interleukin-1β

IL-12 – interleukin 12

MCP-1 – monocyte chemotactic protein-1

NO – nitric oxide

ApoB-LP – apolioprotein B-containing lipoprotein

VLDL – very low-density lipoprotein

M-CSF – macrophage colony stimulating factor

PDGF – platelet-derived growth factor

FGF – fibroblast growth factor

SMC – smooth muscle cell

MMP – metalloproteinase

DC – dendritic cell

LCAT – lethicin-cholesterol acyltransferase

PLTP – phospholipid transfer protein

HL – hepatic lipase

EL – endothelial lipase

RCT – reverse cholesterol transport

PON1 – paraoxonase 1

LpPLA2 – lipoprotein-associated phospholipase A2

NO – nitric oxide

eNOS – endothelial nitric oxide synthase

VCAM-1 – vascular cell adhesion molecule 1

ICAM-1 – intracellular adhesion molecule 1

PL – phospholipid

TG – triglyceride

SLE – systemic lupus erythematous

ESRD – end-stage renal disease

SAA – serum amyloid A

CRP – C-reactive protein

LXR – liver X receptor

CYP7a – cholesterol 7α hydroxylase

RXR – retinoid X receptor

LXRE – liver X receptor response element

SREBP-1c – Sterol regulatory binding-element 1c

PPAR – peroxisome proliferator-activated receptor

NPC1L1 – Neimann-Pick C1-like 1

TICE – trans-intestinal cholesterol excretion

ACC – acetyl-CoA carboxylase

FAS – fatty acid synthase

SCD-1 – stearoyl Co-A desaturase-1

ChREBP – carbohydrate response element binding protein

AP-1 – activation protein-1

GPS2 – G-pathway suppressor suppressor 2

APR – acute phase response

NCoR – nuclear receptor corepressor complex

SMRT – silencing mediator of retinoic acid and thyroid hormone receptor

CORO2A – coronin 2

CHAPTER 1: Introduction

This introduction is a comprehensive overview of topics that I found to be relevant to my PhD studies and topics that repeatedly surfaced as I was trying to understand the various phenomena that I was uncovering in my work. The task of writing an introduction for my thesis therefore became the opportune time for me to really explore and become more knowledgeable in the various topics that I found to be relevant and interesting. My thesis work has focused on the role of the Liver X Receptors in regulating cholesterol metabolism and the reverse cholesterol transport pathway and the effect of perturbing this activity on the development of atherosclerosis. Moreover, I discovered that LXRs can influence HDL levels and particle size as well as increase their functional activity, and importantly, that effect may be influenced by dietary cholesterol. Thus, in this introduction I have attempted to provide a comprehensive overview of the following topics which I consider relevant to my studies: 1) cholesterol metabolism and the effects of diet on blood cholesterol levels; 2) Historical perspective of atherosclerosis and the development of the various hypotheses of atherogenesis; 3)the molecular basis of atherosclerosis; 4) the athero-protective effects of high density lipoprotein (HDL) and factors that lead to its dysfunction; and 5) Liver X Receptors and their potential anti-atherogenic activities. For those looking for a condensed version the most relevant to this work would be best served to start at the HDL section (p.41).

1.1 Cholesterol – an essential molecule

Cholesterol was first isolated from gallstones by physicians in 1789 during the French Revolution, and since that time its complex biosynthesis and metabolism has been extensively studied. Cholesterol is an organic sterol molecule that is an essential component of animal cellular membranes, where it typically accounts for 20-25% of membrane lipid molecules. Cholesterol helps to generate a semi-permeable barrier between cellular compartments and to regulate membrane fluidity. Within the cell membrane cholesterol functions in intracellular transport, cell signaling and nerve conduction and is essential for the structure and function of vesicular endocytosis.

Cholesterol also aids cell signaling by organizing lipid rafts in cellular membranes that function to bring surface receptors in closer proximity to secondary messengers. The metabolites of cholesterol – bile acids and steroids – have important biological roles as signal transducers and solubilizers of other lipids and lipid-soluble vitamins. Moreover, cholesterol is important in the pathogenesis of cardiac and brain vascular diseases and has also been implicated in dementias, diabetes and cancer, as well as several rare monogenic diseases, including familial hypercholesterolemia and lysosomal cholesterol-sphingolipid storage diseases[2, 3]. The biomedical importance of cholesterol in human physiology is undisputable as evident by the degree and variety of pathological states that are associated with either cholesterol deficiency or excess.

Sources of cellular cholesterol

The contribution of *de novo* cholesterol synthesis versus dietary cholesterol for total body cholesterol has been estimated to be 70% and 30%, respectively[4]. This ratio is likely highly variable among individuals, depending both on genetic factors (effectiveness of cholesterol production and absorption) and dietary supply. Since cholesterol is synthesized in the body dietary intake is not a requirement. Yet cellular cholesterol levels must be tightly controlled within a certain physiological range; therefore, under normal conditions there is a balance between *de novo* synthesis and absorption from dietary sources. When dietary cholesterol intake is very low (vegans) its synthesis and absorption is increased. Conversely, when dietary cholesterol is in excess (heavy meat consumption), *de novo* synthesis will be turned off and biliary and intestinal cholesterol excretion will increase.

De novo *cholesterol synthesis* All nucleated cells can synthesize cholesterol from acetyl-CoA via the mevalonate pathway. Production rates vary by cell type and organ function. About 20-25% of total daily cholesterol production occurs in the liver; other sites of increased production include the intestine, adrenal glands and the reproductive organs. Cholesterol biosynthesis (Figure 1.1) consists of a series of elongation reactions of the acetyl-CoA molecule producing farnesyl pyrophosphate, which is then converted to squalene - the first four-ring sterol in the pathway. The pre-squalene steps of cholesterol biosynthesis produce isoprenoids, farnesyl pyrophosphate, and geranylgeranyl pyrophosphate, which are critical for membrane anchoring of signaling proteins. Squalene epoxidase and lanosterol synthase catalyze the conversion of squalene to a relatively inert sterol, lanosterol, which has been implicated in regulating cellular stress pathways[5] and the subsequent steps also produce series of precursors possessing various biological activities. The final product of the pathway, cholesterol, can be incorporated into cellular membranes and/or subjected to oxidative modifications in the "tail" and "B" ring of the molecule to produce bile acids, steroid hormones, and vitamin D[6, 7]. Arresting cholesterol biosynthesis in the pre-squalene steps is universally lethal in all eukaryotes due to disruption of critical membrane-based signaling. In contrast, mutations distal of squalene are viable but produce several developmental defects[8]. Therapeutic trials of cholesterol supplementation in patients with inborn errors of cholesterol biosynthesis have only shown modest improvements[9, 10], thus corroborating evidence that accumulating cholesterol precursors can exert unique biological activities.

The rate-limiting step in cholesterol synthesis is the conversion of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) to mevalonate by HMG-CoA reductase. HMG-CoA

reductase and several other enzymes integral to later steps of cholesterol synthesis are integral endoplasmic reticulum (ER) membrane proteins. The ER also contains enzymes for several key cholesterol processing steps, such as hydroxylation to generate oxysterols (with hydroxyl groups typically added to sterol side-chain carbons). Oxysterols are found in cells in minor amounts typically 1:1000 compared with cholesterol[3]. Hydroxyl groups render sterols more hydrophilic allowing oxysterols to move more freely than cholesterol in the aqueous cytoplasmic environment and function as potent signaling lipids.

Regulation of Cholesterol Synthesis The mechanisms that maintain cholesterol homeostasis are quite sensitive to and directly regulated by the level of cholesterol present in the cell. Greater cholesterol intake results in reduced endogenous synthesis whereas lower dietary consumption of cholesterol increases endogenous production. The main regulatory mechanism is the sensing of intracellular cholesterol within the endoplasmic reticulum by sterol regulatory element-binding protein (SREBP) - 2. If intracellular cholesterol is low SREBP-2 is activated by a cleavage event that frees SREBP-2 from the ER membrane allowing it to migrate to the nucleus where it functions as a transcription factor inducing the expression of many genes involved in lipogenesis. Importantly, SREBP-2 increases the transcription levels of low density lipoprotein (LDL) receptor and HMG-CoA reductase. Thus, a drop in the intestinal supply of absorbed dietary and biliary cholesterol results in reduced hepatic cholesterol levels, which in turn increases LDL receptor synthesis and the upregulation of HMG-CoA reductase; the first to sequester more cholesterol from the circulation and second to increase endogenous synthesis.

Dietary Cholesterol and absorption The majority of cholesterol derived from the diet comes from animal fat as plants contain very little cholesterol. Consequently all foods containing animal fat contain some degree of cholesterol and the major dietary sources, which are prevalent in westernized societies, include cheese, egg yolk, beef, pork, poultry, fish and shrimp[11]. Absorption of cholesterol by the small intestine is critical for the body to maintain cholesterol homeostasis[12]. There are two primary routes for cholesterol to enter the digestive tract – dietary intake and biliary secretion into the duodenum. In a typical Western diet daily cholesterol intake is approximately 300-450 milligrams and complements the 800 -1400 milligrams of endogenous cholesterol from bile. In total, about 800-2000 milligrams of cholesterol is available for absorption from the intestinal lumen per day. Niemann-Pick C1-like 1 protein (NPC1L1) is a cholesterol transporter localized on the luminal side of enterocytes and regulates the majority of dietary sterols and cholesterol absorption[13]. It was recently discovered that ezetimibe, a widely prescribed drug for lowering blood cholesterol levels, inhibits NPC1L1 activity, reducing cholesterol absorption by approximately 70%[14].

Non-esterified cholesterol from bile and food intake is incorporated into miscelles allowing the diffusion of cholesterol via various membrane receptors into enterocytes[15]. Once in the enterocyte cholesterol is esterified and incorporated into chylomicrons along with dietary triacylglycerol (TAG), microsomal transfer protein and apoB48. Chylomicrons are responsible for the bulk transport of cholesterol from the intestine to the circulation via the lymphatic system. Chylomicrons are quickly metabolized in the bloodstream by Lipoprotein Lipase which releases TAGs for energy production. The

cholesterol-enriched chylomicron remnants are then taken up by the liver for further processing.

Is excessive cholesterol in the diet harmful?

Numerous studies in humans have examined the effects of dietary cholesterol on total plasma and lipoprotein cholesterol concentrations. The majority of studies have reported a linear relationship between changes in cholesterol intake and serum cholesterol concentrations; yet many others, including a meta-analysis of 27 controlled metabolic feeding studies of added cholesterol, have indicated that there is little effect on serum cholesterol values when cholesterol consumption is within the normal range of 0-400 mg/dL. In this meta-analysis, the incremental serum cholesterol response to a given amount of dietary cholesterol diminished as baseline serum cholesterol intake increased[16]. A number of studies have also reported that a rise in serum cholesterol levels due to increased cholesterol consumption is attenuated by diets low in saturated fat, high in polyunsaturated fat, or both[17-19], although this effect has not been observed by others[20, 21]. The response of serum cholesterol levels to dietary cholesterol is highly variable among individuals, which may explain the equivocal results from the various studies in humans[16]. Yet the relative responsiveness of serum cholesterol levels appears to be relatively stable within individuals [22, 23] and is associated particularly with saturated fatty acids [24]. Furthermore, intrinsic differences in intestinal cholesterol absorption[25], suppression of hepatic cholesterol synthesis by dietary cholesterol [21, 26-28] and LDL catabolism [26, 29] may all contribute to the observed variation in serum cholesterol response to diet.

Dietary response and genetics A growing number of genetic factors have been identified that may be responsible for a significant proportion of the inter-individual variation in response to dietary cholesterol. One informative case is that of the Tarahumara Indians of Mexico who are habituated to a very low cholesterol, low fat diet and have low plasma cholesterol levels. In response to high cholesterol feeding these individuals have decreased cholesterol biosynthesis as would be expected, however, their intestinal absorption of cholesterol remains unchanged and there is little to no change in their serum cholesterol values. Thus the Tarahumara are one example of a group of individuals who have a reduced ability to absorb dietary cholesterol and therefore may be protected against diet-induced changes in serum cholesterol levels[30].

Variations in several genes have been associated with altered responsiveness to dietary cholesterol. Individuals with the common E4 polymorphism of the apolioprotein (apo)E gene have increased absorption of dietary cholesterol[31]. The observation that cholesterol absorption and bile acid formation is perturbed in *apoE*$^{-/-}$ mice[32] supports the idea that the *apoE* gene plays an important role in modulating dietary cholesterol responsiveness in humans. The A-IV-2 variant allele of the apolioprotein A-IV gene has also been found to attenuate the plasma cholesterol response to dietary cholesterol[33]. Finally, defects in the genes encoding ABCG5 and ABCG8, two heterodimeric cholesterol transporters found in the intestine, leads to the increased absorption of cholesterol and plant sterols[34]. This finding suggesting that more common variants of the ABCG5 and ABCG8 genes found in humans may also contribute to the variation in responsiveness to dietary cholesterol.

Dietary cholesterol and cardiovascular disease The connection between cholesterol intake and serum cholesterol levels has been of great interest since early animals studies demonstrated that dietary cholesterol causes arterial lesions - an effect mediated largely through elevations in blood cholesterol levels[35-39]. These findings led to the "diet-heart" hypothesis that dietary saturated fat and cholesterol raises blood cholesterol levels therefore increasing the risk of cardiovascular disease (CVD). The diet-heart hypothesis was then quickly followed by several short-term feeding studies placing cohorts of participants on both low and high-fat diets and measuring the change in their serum cholesterol concentrations. Unfortunately, there were significant flaws in study design, data analysis and use of controls in most of these studies[40]. Nevertheless, the idea that saturated fat was the primary dietary influence on blood cholesterol was widely accepted, and Dr. Ancel Keys, a pioneer in cardiovascular disease research, proposed a formula to predict changes in blood cholesterol levels based on the amounts and types of dietary fats [41]. In 1957, Dr. Keys implemented the landmark Seven Countries Study[42], which was the first to systematically examine the relationship among, diet, lifestyle, risk factors and coronary heart disease and stroke in populations differing in diet, especially levels of dietary fat. Field surveys began in the US, Italy, Greece, Yugoslavia, the Netherlands, Finland and Japan with follow-ups for morbidity and mortality made every 5 to 10 years.

The Seven Countries Study is said to have "proved" the diet-heart hypothesis, that elevated blood cholesterol levels and intake of saturated fatty acids is a major contributor to cardiovascular disease risk in populations. Subsequent researchers had criticized Dr.Keys' conclusions and pointed out serious flaws and inconsistencies, however, they were largely ignored and the theory that saturated fats cause heart disease became widely

accepted. The seven countries in Dr.Keys' study were said to have been chosen for their contrasting dietary patterns and the relative uniformity of their rural populations. However, Dr. Keys had chosen only those countries where both saturated fat consumption and heart disease were high. He ignored the other 16 countries with similar diets but low rates of CVD. Had the final analysis included this additional data the association between diet and CVD would have been much less clear and likely would have led to different dietary advice[43].

Is there an association between dietary exposures and CHD? The Bradford Hill criteria is a set of guidelines widely used by epidemiologists to systematically evaluate whether a causal link between an exposure of interest and health outcome exists[44]. In the first systematic review of the literature, Anand and colleagues[45] used the Bradford criteria to investigate the causal link between dietary factors and coronary heart disease (CHD) by compiling the results from all eligible prospective cohort studies (147) and randomized controlled studies (43) published between 1950 and 2007. In applying the Bradford Hill criteria, they identified strong evidence of a causal relationship for protective factors, including vegetables, nuts, monounsaturated fatty acids and a Mediterranean diet, and harmful factors, including trans-fatty acids, foods with a high glycemic index or load, and a Western diet. Among these dietary exposures, however, only a Mediterranean diet has been studied in randomized control trials and is significantly associated with a decreased risk of CHD. In addition, Anand et al. found modest evidence to support a causal relationship for the intake of fish, ω-3 fatty acids, folate, whole grains, dietary vitamins E and C, fruits, and fiber, and weak evidence of causation for intake of supplementary vitamin E and ascorbic acid, saturated and polyunsaturated fatty acids and total fat, α-

linolenic acid, meat, eggs, and milk. The modest or weak evidence of these dietary exposures were mostly consistent with results of randomized control trials, although such trials have yet to be conducted for several of these factors. Taken together, these findings indicate that a causal relationship exists only between a few dietary exposures and CHD and the evidence for most individual nutrients or foods is too modest to be conclusive.

The Dietary Guidelines for Americans were revised in 2005[46] to reflect the general consensus that reducing saturated and trans-fatty acid consumption and increasing fruit, vegetable, polyunsaturated fatty acid and whole grain intake is beneficial for CHD[47, 48]. These assumptions were derived from the wealth of epidemiologic studies, however, little direct evidence from randomized controlled trials support these recommendations. Despite the lack of adequate data, evidence-based recommendations derived from cohort studies have been advocated[49]. Unfortunately, it is now becoming clear that the dietary advice to limit the intake of fat may have resulted in the increased consumption of carbohydrates, which can have adverse effects on CHD risk factors, including raising plasma triglycerides and lowering high-density lipoprotein (HDL) cholesterol levels[50]. Indeed, metabolic studies have shown that higher glycemic index scores are associated with coronary risk factors, including a rise in serum triglycerides and lower HDL-C levels. More recently, the lack of benefit of diets of reduced total fat has been established[51], and the evidence supporting the adverse effect of trans-fatty acids on cholesterol levels[52] and CHD[53-56] has increased.

There is a very strong causal link between CHD and particular dietary patterns. Population-based cohort studies have demonstrated that a quality diet (high in whole grains, fruits and vegetables with modest lean meat consumption and limited saturated

fats) is protective against CHD and all-cause mortality[57-61]; these benefits are additive as well with other lifestyle modifications aimed at promoting well-being. Furthermore, the Lyon Diet Heart Study demonstrated that a Mediterranean diet reduces mortality in patients who already have CHD[62]. Studying dietary patterns has the advantage of accounting for the complex interactions and compounding effects of the multiple nutrients present in the diet and more accurately represents food and nutrient consumption[63]. It is now widely accepted that studies aimed at identifying the ideal "Heart-Diet" should test various dietary patterns as opposed to single macromolecules; as had been the precedence for much of the findings which led to the current dietary guidelines.

1.2 Atherosclerosis

More than 100 years ago, German pathologist Virchow observed that the arteries of patients dying from occlusive vascular disease were often thickened and irregular and contained a yellowish fatty substance later identified as cholesterol. He called these plaques atheroma, derived from the Greek word for 'porridge' and postulated that this atheroma contributed to death from cardiovascular causes. Virchow was correct and it is now common knowledge that atherosclerosis is the key contributor to cardiovascular disease (CVD). CVD is currently the leading cause of mortality in developed nations and is it projected that death from CVD will soon reach that status worldwide[64].

Atherosclerosis has been perceived as a disease of modern times because of its associations with the sedentary lifestyles and poor diets present in many of the societies of the current era. Yet, a recent multi-national study spearheaded by Thompson and colleagues tells a different story. Thompson et al. reportedly found evidence of

atherosclerosis in mummified remains representing four geographic areas and spanning more than 4,000 years: ancient Egyptians, ancient Peruvians, Ancestral Puebloans of southwest USA and the Unangan of the Aleutian Islands in present-day Alaska. Overall, probable or definite atherosclerosis was found in one third of the mummies in all four populations. Those with atherosclerosis were about a decade older at the time of death (43 versus 32 years of age); yet, the reason(s) for the presence of atherosclerosis in all four of these populations remains unclear. All four groups were fairly active, none were vegetarian, and all shared common dietary elements. The authors concluded that the presence of atherosclerosis in these pre-modern humans indicate that atherosclerosis is an inherent component of human aging and influenced more so by genetic predispositions and/or other risk factors such as smoking than by any specific diet or lifestyle[65].

Historical perspective

Atherosclerosis research began in earnest at the start of the 19th century as physicians began to investigate the vascular changes that they observed in their patients. In 1829, French surgeon and pathologist Jean Lobstein first introduced the term "arteriosclerosis" to describe the calcified arterial lesions he found in diseased blood vessels. Further studies revealing inflammatory components within these lesions ignited a debate between medical researchers as to whether inflammation was a contributing factor or a secondary effect in atherogenesis[66]. That the immune system does in fact play a key role in atherosclerosis would not be widely supported by the medical community until the end of the 20th century. In 1910, improvements in microscopy allowed German chemist Windaus to describe the composition of the atherosclerotic plaque as consisting of calcified connective tissue and cholesterol[67]. Three years later, Anitschkow and Chaltow

showed that feeding rabbits cholesterol enriched diets produced atheromatous disease similar to what was found in humans[68]. Together, the findings of Windaus and Anitschkow and Chaltow led to the formation of the "lipid hypothesis" postulating that increased serum cholesterol levels drive atherosclerosis. This theory was widely accepted and heavily influenced many early studies aimed at understanding the effect of diet and serum cholesterol on atherosclerosis.

By 1910, heart disease became the number one killer in America, and except for a brief period following the Great Influenza epidemic of 1918, it has remained the most common cause of death in the United States. During the first half of the 20th century, the percentage of deaths due to cardiovascular disease increased substantially across all age groups, in both sexes, and in all races. Indeed, by the 1950s cardiovascular disease accounted for more than 50% of the nation's mortality. A rise in life expectancy due to better treatment of infectious diseases, the increased prevalence of smoking, more sedentary lifestyles, and changes in diet are all factors believed to have contributed to the increase in deaths from CVD over the first half of the century.

The end of World War II brought more attention and financial support to domestic issues, including health, and the American government recognized the enormous toll of cardiovascular disease; thus, the National Heart Institute was created in 1948. The post-war advances in mechanical engineering and electronics seemed well suited for studying the cardiovascular system, as cardiovascular disorders as characterized by pathologies of hemodynamics or electrical function. The initial efforts of the National Heart Institute were quite modest due to limited funding; however, among its most effective actions was the organization of the Framingham Heart Study in 1949,

one of the first and most well-known epidemiological studies of chronic disease. This study set precedence for cardiac epidemiology and findings from the Framingham Study have been responsible for much of the medical guidelines for assessing and addressing cardiovascular disease risk in the clinic[69].

In 1961 the first report from the Framingham Heart Study appeared[70]. This 6 year follow-up report established the concept of risk factors for cardiovascular disease, and hypertension and hypercholesterolemia were identified as major contributors. These findings prompted the National Heart Institute and American Heart Association to promptly establish guidelines and develop both professional and public educational campaigns in an effort to reduce these risk factors in the US. The Framingham study revealed a negative association between cigarette smoke (the 3rd most important risk factor) and cardiovascular disease, which led to the Surgeon General's landmark 1964 report that for the first time in the US publicly denounced the effects of cigarettes on health. It is difficult to determine the relative contributions that the advancements in prevention, diagnosis and treatment made in the fight against cardiovascular disease, however, the reward of the massive campaign efforts was a steady decline in the age-adjusted mortality from heart disease from its peak in 1963[71]. The downward trend in CVD mortality rate parallels a similar decline in Americans with hypercholesterolemia (plasma cholesterol >240 mg/dL) from 34% to 19% between the years of 1962 and 1994[72]; thus providing strong evidence that higher LDL-C levels contributes to heart disease morbidity and mortality.

Discovery of statins Initial studies suggesting a link between serum cholesterol levels and atherosclerosis led to the hypothesis that reducing circulating cholesterol by blocking

endogenous production would reduce the risk of disease. However, early attempts to block cholesterol synthesis failed mainly because of negative side effects attributed to the accumulation of toxic substrates upstream of the inhibited enzyme[73]. HMG-CoA reductase is the rate-limiting enzyme in the cholesterol biosynthetic pathway. In contrast to desmonsterol and other late-stage intermediates, HMG-CoA is water soluble and can be metabolized through alternative pathways when HMG-CoA reductase is blocked; therefore, no potentially toxic precursors are generated. The first HMG-CoA reductase inhibitor, ML236B (compactin), was discovered by Japanese microbiologist Akira Endo during a search for antimicrobial agents[74, 75]. Compactin was quickly shown to lower plasma cholesterol levels in rabbit[76], monkey[77] and dog[78]. Following the positive results in animal models, compactin was quickly developed for human use and shown to effectively reduce plasma total and LDL cholesterol in patients with heterozygous familial hypercholesterolemia[79, 80].

Prior to the introduction of the first HMG-CoA reductase inhibitor, Lovastatin, into the clinic in 1987 the lipid lowering therapies were limited essentially to dietary changes, bile acid sequestrants, nicotinic acid (niacin), fibrates and probucol. All of these treatments however had limited efficacy or tolerability or both. As described earlier, dietary changes tolerable to western societies produce little to no change in total and LDL cholesterol [43, 45]. The bile acid sequestrants (cholestyramine and colestipol), are resins that bind bile acids in the gastrointestinal tract and sequester them from circulation. These compounds are moderately effective at lowering LDL cholesterol; however, since they are not systemically absorbed they cause many gastrointestinal side effects that result in low medication adherence by patients[81]. The fibrates are well tolerated

peroxisome proliferator-activated receptor α (PPARα) agonists with multiple mechanistic functions that synergize to modulate lipoprotein concentrations. Fibrates are now used as combination therapy with statins as clinical trials do not support their use as monotherapy agents. Although less effective at lowering LDL cholesterol, by increasing HDL levels and substantially lowering triglycerides, fibrates appear to improve dyslipidemia in certain patient populations, particularly patients with metabolic syndrome[82]. Probucol, originally developed as an antioxidant, was found to increase LDL catabolism effectively lowering serum lipid levels in patients with Familial Hypercholesterolemia[83, 84]. Yet in other patients the small reduction in LDL cholesterol was offset by a decrease in HDL levels[85, 86]. Because of the strong inverse correlation between HDL cholesterol levels and CHD risk, clinical trials with Probucol were halted.

When Lovastatin became available for prescription large reductions in serum cholesterol was finally achievable. Lovastatin at a maximal dose of 80 milligrams daily produced a mean reduction in LDL cholesterol of 40%[87-90], a reduction never before seen with previous therapies. Other similar compounds quickly followed Lovastatin into the clinic and the class of HMG-CoA reductase inhibitors termed 'statins' was born. All statins produce a qualitatively similar effect on the lipid profile. The mean reduction in LDL cholesterol attainable with the maximal recommended dose of different statins ranges from 35 to 55%. Early studies using quantitative angiography[91-95] or ultrasound[96, 97] showed that statins slowed the progression of atherosclerosis; however, the effects were quite small. It would be another decade before statins were determined effective agents for reducing the risk of cardiovascular events in patients who already had CHD

(secondary prevention) [98-100] as well as in those who did not (primary prevention) [99, 101, 102].

Questioning the lipid hypothesis: why don't statins cure cardiovascular disease?

The medical community was optimistic following the introduction of statins as the standard of care for cardiovascular disease. Statins finally allowed patients to achieve desirable serum cholesterol levels, and physicians hoped that statin therapy coupled with aggressive risk management would drastically cut morbidity and mortality from cardiovascular disease. While risk factor reduction and statin therapy did ameliorated the risk of vascular complications, patients still remained at a high risk for future adverse cardiovascular events. Data from a meta-analysis including 90,000+ patients in 14 randomized trials showed that one in seven treated patients experienced events over five years[103, 104] and further lowering of LDL cholesterol with maximal doses of statins did not eliminate this residual risk[105, 106]. These findings led researchers to focus their efforts on identifying other interventions that could reduce this residual risk among patients receiving optimal therapy. Moreover, the prevalence of residual risk in these individuals led investigators to question the inclusiveness of the lipid hypothesis. By the mid to late 1990s it was widely accepted that treating heart disease was going to be more complicated than merely targeting serum lipid levels. This section will discuss a few of the key theories - the oxidative modification hypothesis, the infectious hypothesis and the atherogenic dyslipidemia hypothesis - that have evolved over the years to help explain atherogenesis and why it is not just about blood lipid levels.

The "oxidative modification" hypothesis of atherosclerosis

The defining characteristic of early atherosclerotic lesions in both animals and humans is the lipid-loaded macrophage "foam cell". These cells originate as circulating monocytes that have infiltrated the sub-endothelial space of the arterial wall where they engulf excessive cholesterol. Researchers postulated, therefore, that studying how arterial macrophages process cholesterol might uncover mechanisms that could be therapeutic targets for cardiovascular disease. Beginning in 1979, Joseph Goldstein and Michael Brown, two University of Texas scientists, decided to study macrophage metabolism *in vitro*[107]. In an interesting first series of experiments they observed that macrophages from patients with homozygous Familial Hypercholesterolemia, who express essentially no functional LDL receptors, were equally effective at accumulating cholesterol as macrophages from patients with normal LDL receptors[107]. Thus, Goldstein, Brown and collaborators speculated that the LDL was altered prior to engulfment and taken up by an alternative macrophage receptor. Indeed, they found that even at very high LDL concentrations, the ability of mouse peritoneal macrophages to take up native LDL was significantly reduced and foam cell formation attenuated[107]. The group then tested a variety of LDL modifications for a mechanism that would turn native LDL into an atherogenic form capable of inducing foam cell formation *in vitro*. Treatment of LDL with acetic anhydride yielded acetylated LDL which bound to macrophages with high affinity and led to increased accumulation of intracellular cholesterol[107]. The macrophage receptor responsible for recognizing and binding acetylated LDL was later cloned and characterized as Scavenger Receptor A (SRA)[108]. The acetylation of LDL however does not occur *in vivo*; therefore the search continued for the biological ligand for SRA as well as the mechanism responsible for LDL modification *in vivo*.

In 1981, Henriksen and colleagues[109] discovered that co-culturing native LDL and endothelial cells induced changes in the LDL that allowed it to be recognized with high affinity by peritoneal macrophages. Later studies revealed that incubation with endothelial cells led to the oxidative modification of LDL that permitted its recognition and rapid uptake by macrophages thereby inducing foam cell formation[110, 111]. Together, the findings of Goldstein, Brown and collaborators and Henriksen and colleagues led to the "oxidative modification hypothesis" of atherosclerosis, stating that production of modified, pathogenic LDL particles initiates and promotes atherosclerosis. This theory expands upon the original lipid hypothesis by providing a mechanistic explanation for the relationship between atherosclerosis and increased serum LDL levels.

Molecular basis of the oxidative modification hypothesis The Steinberg laboratory was one of the first groups to investigate the oxidative modification hypothesis *in vivo*. This group found that LDL extracted from human and rabbit atherosclerotic lesions resembled LDL that had been oxidized *in vitro*. Importantly, Steinberg et al. also demonstrated that LDL from the lesion, but not from the vessel intima or plasma, was chemotactic for monocytes similar to LDL that had been oxidatively modified *in vitro*. Together, these findings supported the hypothesis that LDL in the lesion is oxidatively modified, which recruits monocytes into the vessel wall and promotes foam cell formation[112].

Oxidized LDL (oxLDL) binds with high specificity and affinity to plasma membrane receptors, including SRA, SRB (CD36) and lectin-like oxLDL receptor[113]. In contrast to the LDL receptor that is down-regulated when intracellular cholesterol levels rise, the expression of the oxLDL receptor is not regulated by cholesterol content, allowing the progressive accumulation of cholesterol[113]. The biological properties of

oxLDL have been well characterized *in vitro*. Some of these properties include inducing the expression of monocyte chemotactic factors and vascular adhesion molecules; promoting apoptosis of macrophages and endothelial cells; increasing cytokine production by macrophages; and lastly, raising the levels of oxidation-specific antibodies in circulation[114]. These effects of oxLDL, however, have been primarily studied in cell culture; the role of oxLDL *in vivo*, and especially in humans, is still not well understood.

Vitamin E (an anti-oxidant) treatment in animal models of atherosclerosis attenuates disease development, thereby supporting the oxidative modification hypothesis of atherogenesis[115-118]. Importantly, the protective effects of vitamin E treatment is lost in mice deficient for 12/15-lipoxygenase, an enzyme believed to play a major role in LDL modification within the arterial wall[119]. Likewise, atherosclerosis is accelerated upon the delivery *in vivo* of the 15-lipoxygenase gene[120]. Thus, the protective effects of vitamin E, at least in animals, reside in its ability to block LDL modification. Unfortunately, the results of vitamin E intervention in primary and secondary prevention trials in subjects with or without diagnosed cardiovascular disease have been equivocal[99, 121-125]. The discrepancy among the clinical trials and the observational studies suggests that vitamin E supplementation is not effective in cases of pre-existing disease, may only be efficacious when vitamin E deficiency is present[126], or may only be relevant in the setting of oxidative stress[126-128]. To note, the primary prevention studies[99, 121, 123] were conducted in middle-aged individuals. Therefore, it is possible that starting vitamin E therapy at an earlier age could prevent or retard atherosclerosis. In fact, among the most effective approaches targeting cardiovascular disease in adulthood is the prevention or attenuation of atherogenesis in childhood or even earlier during fetal development[129-132].

To date, no studies have been conducted to determine the effect of vitamin E supplementation on atherosclerosis if started at an early age. However, a very recent study has just shown that low-dose and long-term vitamin E treatment initiated at an early age in *Ldlr*$^{-/-}$ mice effectively reduces atherosclerotic lesions and mortality[133]; thus, further studies in humans across the lifespan may be warranted.

The *"infectious" hypothesis of atherosclerosis*

The similarity between atherosclerosis and chronic inflammatory conditions was first described in the 1850s[134], but only more recently has the cellular composition of lesions been characterized. Circulating monocytes and lymphocytes are recruited into the sub-endothelial space in response to chemotactic factors expressed by endothelial cells that are activated by oxLDL and possibly other species. The inflammatory response associated with atherosclerosis will be discussed in detail in a later section.

Chronic bacterial infections, including *Chlamydia pneumonia* and *Helicobacter pylori*, have been associated with heart disease[135]. To note, there are many confounding variables (e.g. socio-economic status) when linking CVD to infections; however, in studies that took these factors into account patients seropositive for *C.pneumoniae* or *H.pylori* were reported to be more likely to suffer from heart disease than seronegative subjects[136]. In addition, *C.pneumoniae* has been detected in aortic[137, 138] lesions in patients and is found more frequently in atherosclerotic plaques than in non-atherosclerotic tissues[139]. Many of the experimental models studying infectious atherosclerosis introduce *C. pneumoniae* into the respiratory system of animals. In many of these studies, *C. pneumoniae* infection accelerated lesion formation, although some studies reported conflicting results[140]. *C. pneumonia*e is an intracellular pathogen and is able to persist

within the vasculature and resist antibiotic therapy[141]. Such infection has been shown to not only promote inflammation and proliferation in host cells *in vivo*[141], but also to reduce the anti-inflammatory properties of HDL[142]. To be atherogenic *C. pneumoniae* requires a robust immune system; knockout of toll-like receptors (TLR) 2 and 4 and myeloid differentiation primary response gene (MyD) 88 in *apoE*$^{-/-}$ mice attenuate disease development[143, 144], suggesting that the host inflammatory response is the key trigger for atherogenesis. In addition, it has also been suggested that pathogens might promote atherogenesis through molecular mimicry between bacterial and self-antigens. For example, T cells reactive to both human and *C. pneumonia* heat shock protein (HSP) 60 were isolated form human plaques, and autoantibodies against mouse HSP60 were identified following infection of mice with *C. pneumoniae* [145, 146].

The gingival plaque associated with periodontal disease (PD) is colonized by a large number of gram-positive and gram-negative bacteria, including *P. gingivalis*, and *Streptoccoccus mutans*, among others. Oral infections are associated with several systemic diseases, such as infective endocarditis and diabetes. Thus, it is not surprising that periodontal disease has been implicated in CVD risk. Indeed, a meta-analysis of the major epidemiological studies has revealed that there is a moderate, positive association between periodontal disease and cardiovascular outcomes[147-149]. Several periodontal organisms have been identified in human atherosclerotic lesions[140]; however, it is unclear if periodontal organisms promote atherogenesis through direct or indirect mechanisms. Some data suggest that periodontal disease increases systemic cytokines and acute-phase proteins, and such an inflammatory state could augment inflammation in the vessel wall and indirectly influence atherosclerosis[150]. For instance, levels of C-reactive protein, a

biomarker of systemic inflammation and linked to CVD, is two times greater in patients with either periodontal disease or CVD than matched controls, and threefold higher in subjects with both periodontal disease and CVD[151]. Interestingly, standard treatment of periodontal disease for twelve months attenuated systemic inflammation and reversed dyslipidemia (i.e. lowered LDL-C and raised HDL-C) in patients with severe periodontitis[152]. The link between periodontal disease and dyslipidemia is unclear, but similar findings have been reported in mouse models. For example, elevated LDL-C and triglycerides and low HDL-C were found in mice with periodontitis, and HDL cholesterol decreased in *apoE*$^{-/+}$ mice following infection with *P.ginvivalis*[153].

Other infectious agents, including *mycoplasma pneumoniae*, *H.pylori*, and *Enterobacter hormaechei* have been detected in human atherosclerotic lesions[140, 154]. The evidence that these agents play a direct role in atherogenesis is weak; however, they may contribute to the "pathogenic burden". It is now understood that infections by bacteria and/or viruses can contribute to atherosclerosis either directly through infection of vascular cells or indirectly by promoting cytokine production and the acute phase response from nonvascular sites[140]. It is likely that no single organism is responsible for the effects of infection on atherosclerosis, but that atherogenesis is influences by the aggregate effects of the pathogenic burden[155]. Indeed, one study found that 75% of patients with coronary artery disease were positive for three of five "atherogenic" pathogens tested[156]. Moreover, pathogen burden and cardiovascular disease risk were significantly associated, even after adjustment for traditional CV risk factors[156].

Antibiotic therapy and cardiovascular disease

The possible association between infection and CVD prompted clinical trials to assess the therapeutic effect of antibiotic therapy. Four large clinical trials [157-160] focused on patients with stable coronary artery disease. None of these studies showed any long-term benefit of antibiotic treatment in patients with established CVD. Both short-term (6 week) and long-term (1 year) interventions failed to provide any beneficial effect in patients. Conversely, short term treatment with clarithromycin actually increased cardiovascular mortality[160]. Such negative results have led some researchers to argue against a pathogenic role for bacterial microbes and infections in atherosclerosis[161] and CVD[162]. Others, however, have disputed these conclusions[163], citing the difficulty of treating chronic chlamydial infections and the ability of *C. pneumoniae* to develop antibiotic resistance in cell culture experiments[140, 164, 165]. Importantly, these trials were conducted in patients with advanced disease; whether antibiotics exert protective activities in patients with early atherosclerosis is unknown. Furthermore, antibiotic treatment might be ineffective due to the complexity of the pathogenic burden thought to contribute to atherosclerosis; not all organisms will be susceptible to the antibiotics used, allowing these agents to further promote atherogenesis[162].

In conclusion, there is convincing data that supports a positive association for specific pathogens in atherosclerosis, particularly *C. pneumoniae* and periodontal organisms[166]. Overall the clinical and experimental data suggest that pathogens contribute to atherogenesis via direct (infection of host cells) and indirect (upregulation of cytokines and adhesion molecules) mechanisms. Hope that antibiotics might be an alternative and/or complementary treatment for CVD has greatly diminished following the recent

failures of several large clinical trials as well as the recognition that the pathogenic burden that may contribute to CVD is complex and thus likely difficult to target.

Atherogenic dyslipidemia

Prospective epidemiological studies have unequivocally demonstrated that LDL cholesterol levels are predictive for a patient's risk of CVD events and that pharmacologically lowering LDL cholesterol reduces CVD risk in many patient populations[167]. These findings supported the use of risk prediction algorithms to identify individuals with elevated LDL cholesterol who were at an increased risk for CVD. These patients were then prescribed target LDL cholesterol goals believed to be attainable through lifestyle and pharmacological interventions[168]. Until the recent change to the cholesterol recommendations that eliminated target LDL-C levels[169], patients with CHD or CHD risk equivalents were challenged to lower their LDL-C level to less than 100 mg/dL. Many patients who achieve these target LDL-C levels, however, are still at risk for CVD events. Moreover, many individuals with normal LDL-C levels nevertheless develop CVD, especially within the older age groups[170]. Therefore, although widely used in clinic because of its utility, LDL-C concentrations is likely a poor representation of CVD risk.

LDL is a heterogeneous group of particles ranging from small, dense, lipid-depleted particles to large, buoyant cholesterol-enriched particles[171]. While there is much controversy surrounding the impact of LDL particle size on atherosclerosis, a number of studies have suggested that small LDL particles are more atherogenic[172-175]. Since small LDL contains less cholesterol per particle, a patient may have a normal LDL cholesterol level but a preponderance of small LDL; thus their increased CVD risk would be missed

if measuring only the concentration of serum LDL cholesterol. The term atherogenic dyslipidemia was first used by Austin and colleagues[176] to describe the risk-conferring plasma lipid profile comprised of a higher proportion of small LDL particles, reduced HDL-C, and increased triglycerides. Atherogenic dyslipidemia is a signature characteristic of patients with obesity, type 2 diabetes mellitus, insulin resistance, and metabolic syndrome[177, 178] and has become an important indicator of increased CVD risk in these populations.

Besides the traditional blood lipid measurements of LDL-C, HDL-C and triglycerides, recent advancements have allowed for better assessment of lipoprotein subfractions. The best established is the measurement of blood apolioprotein (apo) B concentrations. Each non-HDL particle harbors one apoB molecule; therefore, the apoB concentration represents a count of non-HDL particles in circulation. More sophisticated techniques including analytical ultracentrifugation, gradient gel electrophoresis, and nuclear magnetic resonance (NMR) allow for the quantification of particles within each lipoprotein class and subclass; however, there are time and cost barriers that prevent the widespread use of these methods the clinic.

A number of recent studies have now used these lipoprotein subfraction measurement techniques to assess whether any of the subfractions possess prognostic power for CVD or CVD intermediate endpoints such as carotid intima-thickness. Many of these studies reveal that the concentration of small LDL particles is a better predictor of cardiovascular events than LDL-C levels [175, 179-183]. The increased atherogenicity of small LDL particles is thought to be due to a combination of several biological properties that have been observed – small LDL particles are more susceptible to oxidation than

larger particles, therefore more likely to instigate vascular inflammation; they bind more tightly to arterial proteoglycans perhaps allowing them to enter the arterial wall more easily; and in contrast to mid-sized LDL particles, small LDL particles have relatively lower affinity for the LDL receptor, resulting in decreased cellular uptake and more time spent in the circulation where the particles would have prolonged influence on atherogenesis[173].

Studies which report the concentration of small LDL particles to be predictive of CVD endpoints also find that the total number of LDL particles is similarly predictive [175, 179-183] likely because the amount of small LDL and total LDL particle number (LDL-P) is highly correlated. This correlation may be explained by the idea that among individuals with equal LDL-C levels, the same amount of cholesterol distributed among more particles implies that these particles must be smaller. If this is the case, then it is possible that all LDL particles are equally atherogenic; the association between small LDL particles and CVD disease would, therefore, be explained by the increase in LDL-P, rather than the small LDL particles possessing unique atherogenic traits. Regardless of whether the small LDL particle number or LDL-P values are used, either provide prognostic information distinct from the standard LDL-C measurement. Findings from the Framingham Offspring Study, a large, community-based study that stratified men and women by their LDL-C and LDL-P concentrations, reinforced the prognostic value of LDL-P values. Importantly, the authors report that stratification by LDL-P discriminated CVD event-free survival, whereas there was no difference with stratification by LDL-C. In addition, there was a high degree of variability among individuals in the cholesterol content of LDL particles leading to frequent discrepancies between LDL-C and LDL

particle number.[184] This finding, in addition to other studies reporting LDL composition variability among individuals, has led investigators to search for heritable factors that may influence LDL composition.

Genetics and dyslipidemia Complex segregation analyses indicate that atherogenic dyslipidemia has a strong genetic basis likely resulting from the contribution of multiple genes[176, 185, 186]. Genes with variants that have been reported to be associated with LDL size include: *CETP* encoding cholesterol ester transfer protein, which transfers cholesteryl esters from HDL to LDL particles; *LDLR*, encoding the LDL receptor, which regulates LDL uptake; *LPL*, encoding lipoprotein lipase, which is responsible for converting VLDL to LDL; *MTP*, encoding microsomal triglyceride transfer protein, which transfers triglycerides to nascent VLDL within hepatocytes; and the apolipoprotein genes *APOA5, APOB, APOC3, and APOE*, which are constituents of varied lipoprotein particles[185-193]. All of these genes play a role in regulating LDL particle size and composition (as well as other lipoprotein particles) and so may contribute directly to atherogenic dyslipidemia.

Diet and atherogenic dyslipidemia As discussed earlier, the scientific literature is somewhat divided on the role of diet in altering plasma lipid levels. The current notion is that, contrary to what was proposed 30 years ago, dietary saturated fat probably has little to no effect on serum LDL-C. To address the effect of diet on atherogenic dyslipidemia (i.e. the distribution and composition of particles and not just particle cholesterol levels), a group of healthy middle-age men were placed on high-fat, low-carbohydrate and low-fat, high-carbohydrate diets in a crossover study in which they consumed each diet for 6 weeks. The proportions and types of fats (unsaturated vs. saturated, 1:1 ratio) and types

of carbohydrates (simple vs. complex, 1:1) remained fixed in these diets. Across all subjects, there were higher levels of triglycerides and small LDL particles while on the low-fat/high-carb diet compared to the high-fat/low-carb diet. Interestingly, one third of study participants who had normal LDL particles converted to small LDLs when switched from the high-fat/low-carb to low-fat/high-carb diet[194]. Thus, the authors concluded that reducing fat consumption while concurrently increasing carbohydrate intake promotes atherogenic dyslipidemia. This finding was also confirmed in pre-menopausal women[195]. In these studies the changes in fat calories were balanced by reciprocal changes in carbohydrate calories, preventing investigators from determining whether dietary fat or carbohydrates were the major perpetrators of atherogenic dyslipidemia. In a study of 178 overweight men compared on a high-carbohydrate diet versus a low-carbohydrate diet, and the difference in calories being made up in protein and not fat, the subjects had a higher prevalence of small LDL in response to high-carbohydrate feeding[196]. Additional analysis of this study also found that saturated fat content had little to no effect on components of the atherogenic lipoprotein phenotype, consistent with other studies[197, 198].

Follow-up analysis of the Framingham Heart Study also confirmed that fat content in the diet, after multivariable adjustment for carbohydrate intake and other potential confounders, did not significantly affect LDL size or triglyceride levels in either men or women[198]. Taken together, it appears that the type or amount of fat ingested contributes very little if anything to the development of atherogenic dyslipidemia. However, reducing carbohydrate consumption[199] or losing weight[196] has been shown to attenuates atherogenic dyslipidemia (although these effects do not seem to be additive).

Metabolic disorders and Atherosclerosis Until the 1970s, physicians considered atherosclerosis to be a natural pathology associated with aging, and while it's true that CVD and life span expectancy have increased simultaneously over the past century, the more likely reason for the exponential rise in CVD during this time period is the increase in cardiovascular (CV) risk factors, including hypercholesterolemia, cigarette smoking and hypertension[66]. Indeed, in the US and Western Europe reductions in risk factors and improvements in the treatment of CVD have yielded a decrease in age-adjusted cardiovascular deaths, more so in men than in women[64]. Unfortunately, the ongoing obesity epidemic threatens to undermine these gains. Obesity has reached epidemic proportions worldwide[200] and is associated with increased risk of premature death[201]. Central adiposity is associated with increased cardiovascular morbidity and mortality, and this is independent of the association between obesity and other cardiovascular risk factors[202, 203]. Interestingly, even within the normal body mass index range, weight gain during adult life[204], or even childhood and adolescence[205], increases the risk of diabetes and CVD. The increasing rate of childhood obesity now threatens to lower the life expectancy in the United States for the first time in modern history[206] and the American Heart Association has classified obesity as a 'major, modifiable risk factor' for CVD[207].

Obesity is characterized by the chronic overabundance of nutrients and an unbalanced energy expenditure leading to the accumulation of fatty acids in the liver, muscle and adipose tissue. Free fatty acids can either be oxidized or stored as triglycerides; however, in the setting of obesity these pathways are overwhelmed and fatty acid intermediates, such as diacylglycerol and ceramide accumulate[208, 209]. These free fatty acid metabolites can bind TLR4 present in adipocytes and macrophages, thus

triggering innate immunity and initiating potent downstream inflammatory responses through nuclear factor κB (NFκB) signaling pathways as well as others[210]. In addition, obese compared to lean adipose tissue shows increased expression of inflammatory molecules (e.g. TNFα, IL-6, IL-1β and MCP-1)[211, 212].

Obesity and atherosclerosis share several characteristics; traits that are likely responses to the increased inflammation present in both states. Tissue infiltration by macrophages is a signature of both obese adipose tissue as well as atherosclerotic lesions. The recruitment of macrophages by adipose tissue in the obese state resembles the chemotaxis of these cells into blood vessel walls. Although adipocytes themselves secrete a variety of bioactive molecules, the infiltrating adipose tissue macrophages are responsible for most of the inflammatory mediators [213, 214]. Cell death is the second trait shared between obesity and atherosclerosis. Apoptosis of smooth muscle cells in atherosclerotic lesions causes fibrous cap thinning and contributes to plaque weakening and thrombosis[215, 216]. Similarly, the number of necrotic adipocytes in adipose tissue is much greater in obese individuals compared to lean controls[217]. In conclusion, obesity and atherosclerosis share similar characteristics associated with them both being disease of chronic inflammation. Inflammatory mediators and hormones secreted by adipose tissue and/or adipose associated macrophages may directly enhance the atherogenic process within the vessel wall. Furthermore, there is increasing evidence that obesity may also directly promote HDL dysfunction, the mechanisms by which this may occur will be discussed in a following section.

Molecular pathogenesis of atherosclerosis

There are four major steps that result in the clinical manifestation of atherosclerosis. These steps are 1) endothelial activation and inflammation; 2) entrapment and modification of lipoprotein particles and foam cell formation; 3) progression of atherosclerotic plaques by fibrosis, thrombosis, and remodeling; and 4) precipitation of acute events. Acute clinical events, such as myocardial infarction ("heart attack"), unstable angina (chest pain due to heart muscle ischemia), sudden cardiac death, and stroke are generally due to plaque destabilization and thrombosis[218]. Major risk factors for cardiovascular disease can act at more than one step of atherogenesis. For example, hyperlipidemia can contribute to endothelial activation[219]; impair nitric oxide (NO) synthesis[220]; promote foam cell formation (following modification)[221]; activate platelets and increase thrombotic potential (e.g. hyperlipidemia is associated with increased oxidized phospholipids that interact with scavenger receptor CD36 on platelets)[222]; and lead to reversible plaque destabilization (likely due to inflammation associated with hyperlipidemia)[223]. The progression of the atherosclerotic plaque is illustrated in Figure 1.2.

Lesion initiation The first step in atherosclerosis is the expansion of the arterial intima, a normally small space between the endothelium and the underlying vascular smooth muscle cells. The intima fills with lipids, cells, and extracellular matrix in a process that occurs over decades and typically begins in early adolescence. While this process itself is relatively benign thanks to the preservation of the arterial lumen (only if the lumen is occluded by 80% do serious symptoms occur), advanced lesions have the propensity to undergo necrosis which leads to acute, occlusive thrombosis. Atherogenesis is a focal disease process occurring primarily at sites of disturbed laminar blood flow, notably

arterial branch points and bifurcations. Studies of the earliest stages of atherogenesis in humans and animal models indicate that the key initiating step is the accumulation of ApoB-containing lipoproteins (ApoB-LPs) in the subendothelial space of the blood vessel wall[224]. ApoB-LPs consist of a core of neutral lipids, primarily cholesteryl fatty acyl esters and triglycerides, surrounded by a monolayer of phospholipids and proteins. Hepatocytes secrete ApoB-LPs as very low-density lipoprotein (VLDL) particles which are rapidly converted in circulation to atherogenic LDL particles. LDL particles are the predominant atherogenic ApoB-LP in circulation; however, chylomicrons containing dietary lipids can also be converted by lipolysis to atherogenic remnant particles in circulation and may contribute to the mass of atherogenic ApoB-LPs in circulation[225].

Studies in humans and animals show that the movement of LDL particles from the circulation into the vessel wall is determined by both ApoB-LP concentrations and arterial wall permeability. Any change in circulating ApoB-LP levels will quickly affect how much ApoB-LP is delivered into the vessel wall. Vascular permeability is highly variable among individuals, perhaps contributing to genetic susceptibility to CVD. Even in healthy arteries, the permeability of the endothelial layer is variable among individuals by up to 10 fold[226]; so some individuals with low serum LDL-C may experience a relatively high flux of LDL into the arterial wall.

Entrapment of ApoB-LPs in the vessel wall activates overlying endothelial cells to recruit circulating monocytes to the site of retained particles[227, 228]. Activated endothelial cells secrete chemokines that bind the receptors on monocytes and direct their migration. This is an important step in atherogenesis; preventing monocyte entry by blocking chemokine signaling has been shown to prevent or slow down the progress of

atherosclerosis in mouse models[228]. Monocyte derived macrophages in the lesion also secrete ApoB lipoprotein binding proteoglycans[224]. This mechanism likely plays an important role in LP retention in established lesions versus new lesion formation; this effect may also explain why the inflammatory response is persistent in atherosclerosis[229].

Following chemokinesis, monocytes become tethered to and roll along on endothelial cells overlying trapped ApoB-LPs. Firm adherence of monocytes to lesional endothelial cells is directed by interactions between monocyte integrins and endothelial cell ligands. Atherogenesis occurs at sites of blood flow disturbances and platelet aggregation above the lesion may also promote monocyte-endothelial interactions by activating NFκB signaling and expression of adhesion molecules and through the deposition of platelet-derived chemokines on activated endothelium[228, 230]. Firm adhesion of monocytes to the endothelium is followed by their entry into the sub-endothelial space[230]. The majority of monocytes that enter the lesion site differentiate into macrophages while a smaller subset become dendritic cells[231]. This process is driven primarily by macrophage colony stimulating factor (M-CSF) as well as other differentiation factors.

Atherosclerosis progresses as macrophages engulf oxidized LPs, primarily oxLDL, via scavenger receptor SRA and CD36. SRA and CD36 are the primary receptors directing LP engulfment, however, gene targeting studies in $ApoE^{-/-}$ mice indicate that additional mechanisms are also involved in foam cell formation(cite). Once ingested, the cholesteryl esters of the LPs are hydrolyzed to free cholesterol and fatty acids. The free cholesterol then undergoes re-esterification to cholesteryl fatty acid esters

which is the "foam" of the foam cells. This step is important as accumulation of free cholesterol is toxic to cells and may lead to macrophage cell death in advanced lesions.

Lesion growth Upon engulfment of modified LPs, lesional macrophages secrete a variety of cytokines and growth factors, which synergize to recruit monocytes and vascular smooth muscle cells to the site. In particular, interukin (IL)-1β and tumor necrosis factor (TNF) α secreted by lesional macrophages stimulate the local production of platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF), which play a key role in plaque formation. PDGF is secreted by the activated endothelial cells and stimulates smooth muscle cell (SMC) migration into the intima. An important step for SMC migration and proliferation is the secretion of matrix metalloproteinases (MMP) which are responsible for degrading the internal elastic lamina[232]. Lipoprotein lipase, secreted by activated endothelium, also plays a role in promoting SMC proliferation through a complex process[233, 234]. Together, these factors lead to the formation of a dense, fibrous extracellular matrix that comprises the fibrous cap of the atherosclerotic plaque. The principle proteinaceous constituents of the fibrous cap – elastin and collagens – are responsible for the strength of the cap.

Biomechanical analysis demonstrate that a significant amount of circumferential stress is focused on the fibrous cap, which must resist these high stresses to avoid rupture[235, 236]. The stability of the lesion depends on a balance between inflammatory and reparative processes directed by immune cells (predominantly macrophages and T cells) in the lesion. Plaque stability is conferred by the thick, fibrous cap that is able to reduce the circumferential tensile stress and prevent the lipid-rich necrotic core from coming in contact with the blood. While a thick cap confers stability, rupture-prone plaques tend to

have thin, friable fibrous caps[236, 237]. Foam cells produce increased amounts of tissue factor, a powerful pro-coagulant, thereby making the core highly thrombogenic[238]. The lesion is highly dynamic and changes in plaque constituents may predispose some parts of the plaque to rupture as well. For example, regions of high macrophage density are more prone to rupture than those with fewer macrophages present[239]. Moreover, phenotypic changes occurs in plaque vascular SMCs over time so that they are no longer reparative, thereby increasing the likelihood of plaque rupture[240, 241].

Plaque rupture Inflammatory cells erode the fibrous cap through various mechanisms, but the activation of MMPs have been identified as the most significant. Within atherosclerotic lesions, activated macrophages and endothelial cells secrete MMPs, which will weaken the fibrous cap by degrading the matrix proteins, collagen, and other peptides that comprise it. Various hormones, cytokines, steroids and growth factors regulate the expression of MMPs. In addition, activated lesional macrophages secrete cytokines, such as CD40 ligand, which enhance protease production. These proteases can degrade collagen and weaken the fibrous cap as well[242, 243]. CD40 ligand also blocks the ability of SMCs to proliferate[244] and to produce collagen[245] as does other inflammatory cytokines, including IL-1β, TNFα and interferon (INF) γ, which are commonly found in advanced lesions. Together, these effects results in fibrous cap weakening.

Inflammation and atherosclerosis

It is now widely recognized among the scientific and medical communities that atherosclerosis is a chronic inflammatory disease. A significant body of work over the past 20 years has focused on understanding how both the innate and adaptive immune systems contribute to lesion development, progression and thrombotic events[246].

Importantly, inflammation plays a decisive role in the likelihood that the plaque ruptures as well as in the activation of pro-coagulants within the lesion responsible for initiating the coagulation cascade[167]. The outcome of these events is an occlusive thrombus that manifests clinically as an acute coronary syndrome ("heart attack") or ischemic stroke[247, 248]. Evidence that the immune system influences atherosclerosis is the finding that C-reactive protein (CRP), an acute-phase reactant released during inflammatory processes, adds to the predictive power of traditional CVD risk factors[249]. Furthermore, HMG-CoA reductase inhibitors, the most effective therapy for CVD to date, also have anti-inflammatory activity as they have been shown to reduce leukocyte adhesion, accumulation of macrophages, protease production, pro-coagulant and pro-inflammatory mediator expression, antigen presentation, and T-cell activation[250]. Support for the immunomodulatory effect of HMG-CoA reductase inhibitors comes from recent clinical studies. For example, the CARE (Cholesterol And Recurrent Events) trial first demonstrated that statin treatment reduces serum levels of CRP in addition to LDL-C; moreover, the magnitude of risk reduction associated with statins exceeds that expected for lowering LDL-C alone[251]. Findings from retrospective studies also supported the usefulness of targeting CRP with statins in normocholesterolemic patients in both primary[252] and secondary prevention[253] of adverse cardiac events. Prospective findings from the JUPITER trial (Justification for the Use of Statins in Primary Prevention: an Intervention Trial Evaluating Rosuvastatin) showed that statins prevented adverse cardiac events in patients with optimal LDL-C but elevated CRP levels[254].

Innate immunity and the macrophage in atherosclerosis

Monocytes and macrophages are the most numerous leukocytes throughout atherogenesis and they play a critical role in both the initiation and progression of atherosclerotic lesions. Innate immunity is responsible for the recognition of signature molecules, either pathogen-associated molecular patterns (PAMPs) of microbial origin or danger-associated molecular patterns (DAMPs) which are "self" molecules that have become accessible to immune cells following cell injury or death. Important to atherosclerosis, DAMPs can also be "altered self" molecules – originally benign "self" molecules that have undergone modification. Innate immune cells use pattern recognition receptors (PRRs) to recognize DAMPs and PAMPs, directing responses that are normally protective, such as eliminating microbes or removing damaged tissue. Importantly, these early innate immune responses are followed by secretion of chemokines and cytokines that recruit and activate lymphocytes.

Early research in understanding the inflammatory process in atherosclerosis focused on identifying the antigen(s) responsible for the initial inflammatory response. As discussed previously, the possibility that infectious organisms activate this initial response has been considered, however, little evidence supports a primary role[140, 166]. Furthermore, clinical trials have not provided adequate support for the therapeutic use of antibiotics as a primary or secondary prevention for CVD[255]. Similarly, the generation of DAMPs requires a pre-existing injury as they are generally produced during tissue damage. Therefore, while they may contribute to the chronic inflammation of atherosclerosis, DAMPs are unlikely to play a major role in directing the primary inflammatory response of atherogenesis. Laboratory and clinical data indicate that antigens responsible for the initiation of atherosclerosis consist of new epitopes that are

formed as a result of oxidative reactions, such as when oxLDL is formed or cells undergo apoptosis [246, 256-258]. The innate immune system has evolved to remove these oxidized molecules and cells, since they would otherwise be pro-inflammatory and immunogenic[257, 259].

Immune cells normally have limited ability to bind to the endothelium; however, pro-inflammatory stimuli including hypercholesterolemia, hyperglycemia, hypertension, and smoking trigger the endothelial expression of vascular cell adhesion molecule-1 (VCAM-1) and P-selectin and promote the attachment of circulating monocytes and leukocytes[260-263]. The presence of oxLDL in the vessel wall also increases the expression and secretion of chemoattractant factors from endothelial cells, including MCP-1, which direct the migration and entry of monocytes. Indeed, genetic deletion of MCP-1 in *Ldlr*$^{-/-}$ mice reduces monocyte recruitment and decreases atherosclerosis[264]; similar results are true for the genetic deletion of the MCP-1 receptor in *apoE*$^{-/-}$ mice[265]. Experimental studies and human observations also support the involvement of other chemokines in monocyte recruitment into the arterial wall[266-269]. Taken together, these findings support the requirement for innate immune response directing monocyte recruitment, a critical step in atherogenesis.

The inflamed vascular wall overexpresses M-CSF which drives monocyte differentiation into macrophages within the intima[270, 271]. In addition, M-CSF stimulates increased expression of macrophage scavenger receptors responsible for engulfment of ox-LDL by receptor-mediated endocytosis[243]. Lesion monocytes/macrophages also have increased expression of TLRs which activate inflammatory signaling pathways in response to a large number of stimuli, including oxLDL[272, 273] and heat shock proteins

(released from apoptotic cells in the lesion)[274]. Consistent with the role of TLRs and their downstream effectors in promoting atherosclerosis by sustaining inflammation, genetic abrogation of the pathway reduces disease[275, 276]. In addition, atherogenesis is also associated with the selective recruitment of a pro-inflammatory subset of monocytes, Ly-6C^{hi}, which preferentially bind activated endothelium and infiltrate into lesions; serum levels of Ly-6C^{hi} are increased in hypercholesterolemic ApoE$^{-/-}$ mice [277]. Upon lesion formation, resident macrophages proliferate and amplify the inflammatory response by secreting growth factors and cytokines, including TNFα and IL-1β. These two cytokines are important immune modulators responsible for the induction of a broad spectrum of adhesion molecules, chemokines, and growth factors, thereby sustaining the chronic inflammatory state within the lesion[243].

Advanced lesions are characterized by a necrotic core of lipid-filled cells covered by a fibrous cap. The amount and type of interstitial collagen fibers comprising the fibrous cap determined its strength. Inflammation can weaken the fibrous cap by limiting the synthesis of new collagen fibers by SMCs and stimulating the degradation of existing collagen[245]. This response is mediated in large part by the secretion of IL-1β and CD40L by T-cells which induces macrophages to release collagenases that are responsible for the initial degradation of plaque collagen[278-280]. Collagenase expression and activity is increased in regions of the plaque vulnerable to rupture (e.g. core and shoulder)[279, 281] suggesting their role in promoting plaque destabilization and thrombotic events. Indeed, plaque collagen content increases in collagenase-resistant apoE$^{-/-}$ mice[282]. Inflammation also stimulates macrophage foam cells to release proteases that degrade elastin and collagen contributing to plaque evolution and destabilization [283, 284]. The combined effect

of the various proteases released by macrophage foam cells favors fibrous cap remodeling that may lead to plaque rupture[167].

Other innate immune cells involved in atherosclerosis

Neutrophils Despite being important phagocytic cells of the innate immune system and the most abundant type of white blood cell in humans, neutrophils comprise only a minority of the inflammatory cell composition of atherosclerotic lesions[246]. Whether neutrophils directly contribute to atherogenesis is controversial and uncertain. There is some evidence, however, that they may play a role in the initial recruitment of immune cells to the lesion site[285]. Additionally, the degranulation of neutrophils results in the release of a variety of proteins including collagenase, elastase, and proteases which may contribute to plaque destabilization. Indeed, atherosclerotic lesions demonstrate increased markers of neutrophil degranulation and the presence of neutrophil-specific proteases suggesting that neutrophils are playing an active role in the progression of atherosclerosis[286]. It has also been reported that neutrophils co-localize with myeloperoxidase in lesions[287]. Myeloperoxidase generates the reactive oxygen species hypochlorous acid, which contributes to the apoptosis of endothelial cells as well as promoting lesion growth[288]. Myeloperoxidase activity also leads to LDL nitration and lipid peroxidation, increasing the uptake of modified LDL by macrophages[289]. Interestingly, high circulating levels of neutrophils predict myocardial infarction better than any other leukocyte subset, including total white blood cell, lymphocyte, or monocyte count[290].

Dendritic cells Dendritic cells (DCs) are the professional antigen-presenting cells of innate immunity responsible for inducing T-cell responses. As key modulators of the

immune system, DCs likely play a critical role in directing the innate or adaptive immune responses against the altered self-antigens found in atherosclerosis. Indeed, the number of DCs increase in parallel to lesion complexity[291] as does the expression of CD83[292], a marker of DC activation, thus supporting the potential contribution of DCs to atherosclerosis. The influence of DCs in the atherogenic process likely hinges upon their primary function as antigen-presenting cells. Contrary to macrophages that lose their ability to present antigens in the face of cholesterol induced cytotoxicity[293], dendritic cells retain antigen presenting function under conditions typical of atherosclerotic lesions[294]. This ability is probably due to the increased resistance to oxidative stress and cholesterol-induced cytotoxicity characteristic of DCs[294, 295]. Importantly, antigen presentation by DCs primes T-cells and enables T-cell antigen-specific differentiation into effector cells. Efficient T-cell priming requires co-stimulatory signaling from DCs and when this is blocked in athero-prone mice the development of atherosclerosis is attenuated[296].

Adaptive Immunity in Atherosclerosis The second arm of the immunity is the adaptive immune response that is activated in response to antigen recognition by B-cells and T-cells. Antigen recognition drives lymphocyte proliferation and differentiation into effector cells with pro-inflammatory properties meant to be protective against infection. The adaptive immune response, however, also leads to tissue damage, especially when exposure to the antigen is persistent (i.e. atherosclerosis). Indeed, T-cells constitute approximately 10% to 20% of immune cells in advanced human plaques[297] and congregate at rupture-prone sites[298]. Together, these findings suggest that T-cell plays a role in mediating the tissue damage present in late-stage disease.

T-cells enter the vessel wall in response to several different chemokines that all bind the CXCR3 receptor highly expressed on the surface of lesional T-cells[299]. Most of the T-cells in atherosclerotic lesions are of the Th1 subset[300] and recognize antigens presented by macrophages and DCs, including antigens derived from oxLDL[301]. Antigen recognition leads to the clonal expansion of antigen-specific T-cell effector cells[302]. Lesional T-cells secrete INF-γ, IL-2, and TNFα which activate macrophage and vascular endothelial cells and promote inflammation[300]. In addition, compared with non-diseased arterial tissue, there is increased expression of IL-12 and IL-18, Th1 stimulatory molecules, by lesional cells[303]. In addition to Th1 cells, atherosclerotic plaques also contain some cytotoxic Th2 cells and occasional B cells[298]. Although the number of B-cells in plaques is few, their function in antibody secretion may play an important role in atherosclerosis. Indeed, analysis in human subjects reveals both increased plasma titers of anti-oxLDL antibodies and immune complexes containing oxLDL in human lesions[304]. Further evidence for the role of B-cells in atherosclerosis comes from the discovery that antibody titers to heat shock protein 60 (HSP60), which is released from damaged tissue, correlates with cardiovascular disease[305].

The degree to which adaptive immune responses influence the atherogenic process is still under investigation. Studies of atherosclerosis in hypercholesterolemic $ApoE^{-/-}$ or $Ldlr^{-/-}$ mice in which adaptive immunity has been deleted ($Rag^{-/-}$ or SCID background) show that adaptive immunity is not required for atherogenesis, however, its presence affects lesion formation and is largely pro-atherogenic, probably more so at earlier stages[306-310]. Indeed, reconstitution of SCID/ $apoE^{-/-}$ mice with Th1 cells from immunocompetent $apoE^{-/-}$ mice results in lesion growth similar to immunocompotent

apoE$^{-/-}$ mice[308], suggesting that Th1 cells are the primary adaptive immune cells with proatherogenic activity. Additionally, the disproportionately elevated cardiovascular risk for patients with systemic autoimmune diseases[311-313] also supports the role for adaptive immune responses in promoting atherosclerosis.

1.3 High Density Lipoprotein

Early findings that high-density lipoprotein (HDL) cholesterol levels were inversely associated with cardiovascular risk led to massive efforts to define the potential anti-atherogenic activity of this particle. Over a half century of research has focused on defining high-density lipoprotein (HDL) particles by their ability to accept cholesterol from peripheral cells, importantly arterial macrophages, and transport it to the liver for metabolism and excretion. While HDL and HDL-cholesterol (HDL-C) levels have historically been considered one and the same, recent evidence has begun to shine light on the dynamic and variable natures of HDL particles. Human HDL, in fact, is actually a heterogeneous collection of lipoprotein particles with a density of ranging from 1.063 to 1.21 g/ml. Size exclusion column or nondenaturing gradient gel analysis reveals that human HDL has several discrete particle sizes. Ultracentrifugation can separate 2 major density subfractions, HDL_2 (density between 1.063 and 1.125 g/mL) and HDL_3 (density between 1.125 and 1.21 g/ml). Proteomics of HDL is complex[314] but the majority of HDL contains apoA1, which is the most abundant lipoprotein in normal human plasma and comprises approximately 70% of the total HDL protein content. Many HDL particles also contain ApoAII, the second most abundant lipoprotein in HDL. Many of the less abundant proteins associated with HDL are found only in a subfraction of HDL particles[314], thus increasing HDL diversity.

HDL biogenesis

The biogenesis of HDL is a complex and dynamic process involving the formation of nascent particles that undergo lipidation and extensive remodeling in the circulation (Figure 1.3). The generation of mature HDL begins with the synthesis of apoA1 in the liver and intestine. Maturation of nascent HDL particles require interaction with the cholesterol/phospholipid transport ABCA1 for lipidation. Mouse models of tissue specific ABCA1 deficiency demonstrate that hepatic ABCA1 is responsible for the majority of nascent HDL synthesis, while intestinal ABCA1 plays a significant role in HDL maturation[315]. Following ABCA1-mediated lipidation, HDL is enriched in phospholipids and free cholesterol and this particle is the substrate for lethicin-cholesterol acyltransferase (LCAT) which esterifies free cholesterol to cholesteryl ester building up the hydrophobic core and in the process generates larger and less dense HDL particles[316]. LCAT activity is critical for normal HDL metabolism. In humans, the rare genetic disorder of LCAT deficiency is associated with extremely low HDL-C (<10 mg/dL) and apoA1 levels[317]. Loss of LCAT-mediated cholesterol esterification in plasma results in the inability to form mature HDL particles with a cholesteryl ester core and rapid catabolism of circulating apoA1 and apoAII[317]. Interestingly, premature CVD is not evident in LCAT-deficient patients despite the extremely low HDL-C and apoA1 levels[318, 319].

Cholesteryl ester transfer protein (CETP) is another important enzyme that plays a critical role in HDL remodeling. CETP is synthesized in liver and adipose tissue and circulates in plasma bound to lipoproteins. The function of CETP in the plasma is to redistribute hydrophobic lipids packaged within the lipoprotein core (triglycerides and

cholesteryl esters) between HDL and triglyceride-rich apoB-containing particles (VLDL, LDL, IDL, chylomicron remnants)[320]. The net effect of CETP activity on HDL is cholesteryl ester depletion and triglyceride enrichment, with an overall reduction in HDL-C and generation of smaller HDL particles. CETP activity is increased in hypertriglyceridemia[321] and in the post-prandial state[322], perhaps explaining why low HDL-C often appears together with high LDL-C. Despite decreasing HDL-C levels, CETP appears to promote the uptake of HDL-CEs in the liver, arguing that this enzyme might also have anti-atherogenic activity[323]. That CETP plays a critical role in HDL metabolism was conclusively demonstrated by the discovery of a small cohort of CETP deficient patients in Japan who have elevated HDL-C and reduced ApoA1 turnover. Despite a rise in HDL-C levels, however, coronary heart disease was increased among study participants with CETP deficiency[324]. To note, there is discrepancies among the various studies, and in certain cases CETP deficiency is athero-protective, and this effect is seen in parallel with significant increases in HDL-C[325]. CETP is not found in rodents and when it is transgenically expressed HDL-C levels are dose-dependently reduced, and as a consequence, atherosclerosis increases[326]. In contrast, CETP expression is anti-atherogenic in other mouse models more relevant to human pathophysiology (i.e. hypertriglyceridemia)[327]. The protective effects of CETP despite opposite effects on HDL-C suggest that the dynamics of HDL metabolism may be more important than HDL levels in determining the effect on atherosclerosis.

Phospholipid transfer protein (PLTP) is an additional enzyme responsible for the remodeling of HDL in circulation. PLTP circulates bound to HDL and mediates the transfer of phospholipids from triglyceride-rich lipoprotein to HDL and also the exchange

of phospholipids between lipoproteins. The net transfer of phospholipids into HDL results in the formation of larger, less dense particles. Besides phospholipids, PLTP can also mediate the transfer of lipid species, including diacylglycerol, ceramide and lipopolysaccharides[328]. PLTP deficiency has not been found in human, but PLTP knockout mice have a marked reduction in HDL phospholipids, free cholesterol and apoA1[329] due to increased clearance of phospholipid-depleted HDL[330]. Overexpression of human PLTP in mice also results in a 30% to 40% reduction in plasma HDL cholesterol levels[331, 332]. This reduction in HDL-C is accompanied by an increase in preβ-HDL particles[331], which are more rapidly degraded[333], thereby likely contributing to the decrease in HDL levels. Overall, PLTP deficiency as well as PLTP overexpression causes a significant reduction of HDL levels in circulation.

In addition to the actions of LCAT, CETP, and PLTP, a number of lipases present in both the circulation and tissues also contribute to the composition of HDL particles. Three members of the triglyceride lipase gene family of proteins are primarily active in HDL remodeling: lipoprotein lipase, hepatic lipase and endothelial lipase.

Lipoprotein lipase (LPL) is a multifunctional protein that is produced by many tissues including muscle, adipose and macrophages[334]. LPL plays a major role in the hydrolysis of triglycerides from the core of triglyceride-rich lipoproteins, primarily chylomicrons and VLDL[335]. LPL has also been show to promote the exchange of lipids between lipoproteins[336]. It also mediates the uptake and degradation of cholesterol-rich lipoproteins[337-339], and this effect is independent of the lipolytic activity of LPL[339]. Indeed, in cultured human hepatocytes LPL was shown to significantly increase the selective uptake of HDL-CE[340] and this effect is independent of SR-B1[341]. More than 100

mutations have been identified in the human LPL gene. A recent meta-analysis has determined that LPL variants that result in reduced LPL activity are associated with adverse lipid profiles – elevated TGs and lower HDL-C[342].

Hepatic lipase (HL) is lipolytic enzyme that is synthesized in hepatocytes and is secreted and bound to the surface of hepatocytes and hepatic endothelial cells. Lipoprotein components hydrolyzed by hepatic lipase include triglycerides, cholesterol esters, and phospholipids[343]. In contrast to lipoprotein lipase, heaptic lipase does not require interactions with apolipoprotein to be enzymatically active[344] HL hydrolyzes HDL phospholipids and triglycerides[343], and in the presence of CETP converts phospholipid-rich HDL to smaller HDL remnants and lipid-poor or lipid-free apoA1[345]. It has also been shown to enhance HDL-cholesterol ester uptake by hepatocytes, perhaps because of its remodeling effects[323, 344]. In addition, human HDL turnover studies[346, 347] have also suggested that hepatic lipase plays an important role in determining HDL protein fractional catabolic rates, further confirming the role of hepatic lipase in HDL metabolism and HDL-CE uptake. Patients with genetic disruption of HL[348, 349] have moderately elevated HDL-C and knockout[350] and overexpression[351, 352] studies in animals have also demonstrated that HL activity is inversely associated with HDL-C levels.

Endothelial lipase is synthesized and secreted by endothelial cells and has been detected in a variety of tissues, as well as in human atherosclerotic foam cells[353]. Endothelial lipase primarily functions as a phospholipase and exhibits preference for HDL over other lipoproteins[353]. In sum, EL is a negative regulator of plasma HDL-C levels. Overexpression[354-356] and knock out studies[357] in rodents demonstrate that EL activity reduces HDL-C and apoA1 levels due to an increase in the catabolic rate of HDL

apolipoproteins and HDL-CE. It has been posited, therefore, that the hydrolysis of HDL phospholipids by EL destabilizes the particle, resulting in shedding of apoA1 molecules that are rapidly cleared by the kidneys[356]. Consequently, the remaining CE within the EL-modulated HDL particle is more susceptible to SR-B1 mediated uptake[355, 356]. The evidence that EL negatively controls HDL levels in humans comes from several recent GWAS studies[358-360] that identified EL variants associated with HDL-C levels. Further confirming the effects of EL, a meta-analysis across five cohorts demonstrated that loss of function mutations in EL increases HDL-C levels[360].

HDL - the "good" cholesterol

Physicians have long advocated to the public that LDL-C is "bad" whereas HDL-C is "good" based on the early epidemiological findings that high LDL-C increases CVD risk while HDL-C reduces it. The effects of LDL-C and HDL-C on CVD risk are not necessarily related. Indeed, findings in the 1970s and 1980s from follow-up analysis of Framingham Heart Study showed that HDL-C was protective in patients across the strata of LDL-C levels[361, 362]. Further evidence that HDL-C was an important predictor of CVD risk, the incidence of low HDL-C (<35 mg/dL) was approximately three fold greater among men (<60 years old) with premature coronary heart disease than age-matched controls[363]. Genetic disorders characterized by low HDL-C levels are rare and include mutations in or the hypercatabolism of ApoA1, as well as defects in cholesterol transporters such as ABCA1. On the other hand, lifestyle factors such as obesity, physical inactivity, diet, smoking, and the presence of other inflammatory disorders – account for the majority of low HDL-C levels.

Despite the striking epidemiological evidence that HDL-C levels confer protection from CVD, recent human studies have cast serious doubt on the therapeutic

benefit of raising HDL-C levels[364, 365]. Voight and colleagues recently completed a Mendalian randomization study and found that a combined score for the 14 common genetic variants that are associated with HDL-C levels and no other lipoprotein traits was not associated with CVD endpoints[366]. Furthermore, recent drug trials to increase HDL-C, either with CETP inhibitors[364, 367] or extended release Niacin[368], did not show significant beneficial effects on CV outcomes despite raising HDL-C. Interestingly, a mouse model of SR-B1 deficiency on the hyperlipidemic *ApoE*$^{-/-}$ background, in which plasma HDL-C accumulate due to the block in hepatic uptake actually developed more severe atherosclerosis than controls[369]. The accumulating evidence from human and animal studies provides compelling support for the notion that HDL-C in and of itself is not athero-protective. Yet, there is substantial data in mouse models that increased HDL is associated with decreased atherogenesis and increased regression of existing lesions (These studies will be discussed in a following section). Furthermore, human data indicates that HDL particle number is a better indicator of cardiovascular disease risk than HDL-C levels[370]. While this effect may be due merely to raising the amount of HDL particles available to remove excess cholesterol, increasing HDL particle numbers might also impact other functional activities of HDL. It is important to note, however, that the capacity of HDL to accept macrophage-derived cholesterol has recently been shown to be a better marker for CVD risk than HDL-C[371]. Researchers are only now beginning to understand the full spectrum of HDL's function and cargo; thus, more emphasis should be placed on elucidating the other aspects of HDL besides its role in cholesterol transport, as these may be potential therapeutic targets. Nevertheless, it is becoming clear that HDL particle number and function are likely better predictors of CVD risk than HDL-C. While

it may not be feasible to measure such parameters in every patient, new methods that allow such quantification of HDL particle number and function on a large-scale will greatly enhance CV risk reduction.

HDL atheroprotective functions

HDL has been ascribed many atheroprotective activities, including mediating anti-oxidant and anti-inflammatory pathways as well as promoting endothelial cell function and regulating whole body cholesterol transport[372]. Since the accumulation of cholesterol esters by macrophages in the vessel wall is the hallmark of atherogenesis[224] many have ascribed HDL mediated reverse cholesterol transport (RCT) as the major anti-atherogenic function[373-375]. Indeed, as noted above, many recent human studies are indicating that HDL efflux capacity is a reliable indicator of CVD risk[371]. Consistent with atherosclerosis being a dynamic and multi-factorial disease, HDL's other roles likely contribute to its atheroprotective properties, and to what degree these properties play a role in suppressing atherogenesis is currently being investigated.

HDL and reverse cholesterol transport The RCT hypothesis first put forth by Glomset[316] proposes that HDL accepts cholesterol from peripheral cells such as lesion macrophages, and delivers it to the liver where it can be directly excreted into the bile or metabolized into bile salts before excretion. ApoAI, other exchangeable apolipoproteins, and mimetic peptides with the amphipathic helical structure of ApoAI can all accept cellular free cholesterol and phospholipids from ABCA1. In the first step of RCT, macrophage ABCA1 transfers cholesterol and phospholipids to lipid-poor ApoA1 forming nascent HDL (Figure 1.4). LCAT associates with HDL in circulation and promotes its maturation by converting free cholesterol into cholesteryl esters which are immediately sequestered

in the hydrophobic core of HDL[316](Figure 1.4). The importance of ABCA1 mediated cholesterol efflux is appreciated in patients with Tangier's disease. These patients have a loss of function mutation in *ABCA1* and as a result have reduced HDL-C levels and tissue accumulation of cholesterol esters[376, 377]. Not unexpectedly, these patients are at increased risk for developing CVD[378]. Animal studies have further clarified the physiological role of ABCA1. *Abca1*$^{-/-}$ mice have very low HDL-C levels similar to patients with Tangier's disease[379]. Macrophage specific knockout of ABCA1 had no significant effect on HDL-C levels but resulted in increased atherosclerosis, presumably due to a block in macrophage RCT[380]. Thus, macrophage ABCA1 contributes little to the bulk lipidation of ApoA1 and HDL however it is important for atheroprotection. Conversely, hepatic ABCA1 is critical for the initial lipidation of nascent lipid-poor apoA1 particles protecting them from rapid degradation in the plasma as liver specific deletion of ABCA1 reduces plasma HDL-C by approximately 80%[315].

Mature HDL mobilizes additional cholesterol from macrophages and other lipid-laden cells through interaction with ABCG1, SR-B1, or other receptor-independent pathways[381]. SR-B1 binds larger spherical HDL and forms a complex, probably containing a hydrophobic channel, which allows cholesterol transfer to HDL[382]. SR-B1 mediates bi-directional cholesterol flux and allows HDL-cholesterol to enter cells. In circulating monocytes SR-B1 levels are undetectable, but increase upon differentiation into macrophages[383] indicating the importance of this protein for the first step of RCT. ABCG1 is an intracellular transporter which reorganizes the pool of plasma membrane cholesterol thereby facilitating its absorption via passive diffusion by cholesterol acceptors[384, 385]. Nascent and mature HDL particles are equally effective acceptors for

ABCG1-mediated cholesterol efflux[374]. Depending on the cholesterol gradient between the cell membrane and the acceptor, passive diffusion can also promote macrophage cholesterol efflux[386].

Importantly, cholesterol efflux to HDL does not occur in the circulation but within the arterial wall and HDL must cross the endothelial barrier twice to access lipid-laden arterial macrophages and to re-enter the circulation. Indeed, two recent studies have provided *in vivo* evidence that the trans-endothelial transport of HDL into the lymphatic vasculature is a rate-limiting step in reverse cholesterol transport[387, 388]. Endothelial cells can bind, internalize and transport mature HDL via distinct mechanisms regulated by SR-B1, ABCG1 and endothelial lipase[389, 390]. Once in circulation HDL travels to the liver where it deposits its lipid cargo to hepatic SR-B1 (Figure 1.4). In the liver, HDL-derived cholesterol is then secreted into bile by ABCG5/G8, an obligate dimer pair of cholesterol transport proteins, and subsequently excreted via bile into the feces. Alternately, cholesterol can be excreted through a non-biliary pathway termed "trans-intestinal cholesterol export" (TICE) which relies on VLDL targeted to the LDL receptor or another lipoprotein receptor in the small intestine. Intestinal cholesterol is then excreted into the intestinal lumen by ABCG5/G8[391, 392].

***Studying the RCT pathway* in vitro *and* in vivo** Cholesterol efflux assays are useful for studying the first step of the RCT pathway (macrophage to HDL cholesterol transfer), however, proving the RCT hypothesis *in vivo* has been challenging. Rader and colleagues developed an *in vivo* RCT assay system in mice using macrophages that have been labeled with radioactive cholesterol *ex vivo*. These tracer cells are then injected into the peritoneal cavity of mice and the amount of radioactive tracer tracked as it moves into

various compartments, notably the plasma, liver, and feces. As a proof of concept experiment Rader and colleagues demonstrated that RCT can be increased by apoA1 overexpression[393]. The utility of this assay is that it can be modified by using different types of donor cells and/or pharmacologically or genetic manipulating the system. Indeed, the numerous studies in mice using this *in vivo* RCT assay has yielded a wealth of information on RCT in rodents that can be extrapolated to humans. Although numerous HDL turnover studies have been performed in humans[394], the ability to quantify the movement of cholesterol from macrophage to feces has not been worked out. Recently, a study using continuous ^{13}C infusion in humans demonstrated the ability to quantify cholesterol movement *in vivo*[395]. This method, however, does not specifically address the contribution of cholesterol efflux from foam cells, therefore whether this method would be meaningful in the setting of atherosclerosis remains to be seen.

Anti-oxidative properties of HDL HDL function can be measured in several *in vitro* assays. Cell-based and cell-free assays to measure the anti-inflammatory and anti-oxidant activities were first pioneered by Fogelman and colleagues[396, 397]. In a culture system containing endothelial and smooth muscle cells, LDL will undergo oxidation and induce the expression of monocyte chemotactic factors leading to increased monocyte transmigration[397]. The addition of HDL to this culture system can block this response, demonstrating HDL's anti-oxidant and anti-inflammatory activities. The anti-oxidant activity of HDL requires several HDL-associated enzymes, including paraoxonase 1 (PON1), lipoprotein-associated phospholipase A2 (LpPLA2) and LCAT. These enzymes have all been reported to hydrolyze oxidized phospholipids, thereby reducing the oxidative state of lipoproteins particles[372, 398-402]. Additionally, sulfur-containing

methionine residues of apoAI, either singularly or in complex with HDL, have detoxifying activity against phospholipid hydroperoxides rendering them redox-inactive[403].

HDL and endothelial protection. *In vitro* studies have shown that HDL can attenuate the inflammatory response in endothelial cells and induce endothelial repair by promoting endothelial nitric oxide (NO) production. Several different mechanisms have been proposed to account for HDL stimulated endothelial NO synthase (eNOS) increases. Several reports have demonstrated that HDL blocks the detrimental effects of oxLDL on endothelial NO synthase activity[404, 405], and subsequent studies have shown that HDL binding to SR-B1 on endothelial cells directly stimulates NO production[406, 407]. Others have suggested that the HDL binding to endothelial SR-B1 facilitates interaction between HDL-associated lysophospholipids (i.e. sphingosine-1-phosphate) and the S1P3 receptor resulting in increased NO production and vasodilation[408]. Moreover, in an ABCG1-dependent manner, HDL treatment of human aortic endothelial cells prevents oxysterol-induced production of reactive oxygen species, thereby maintaining eNOS activity[409].

HDL and anti-inflammatory activity Fogelman et al.[399, 410] provided some of the early evidence that HDL possessed anti-inflammatory activity *in vitro*. The *in vivo* significance of HDL's role in mediating inflammation was demonstrated by the findings that HDL potently blocks cytokine release in a murine model of endotoxinemia[411]. In this model, HDL was found to bind LPS thereby attenuating immune activation. The anti-inflammatory activity of HDL on vascular endothelial cells is mediated through the HDL-dependent induction of eNOS production as described above. At the cellular level, HDL has been shown to affect such inflammatory processes through the inhibition of cytokine

production, upregulation of critical adhesion molecules, and interference with pro-inflammatory transcription factors such as NFκB[412]. The mechanism by which HDL exerts these effects, however, is poorly understood. NFκB is a major pro-inflammatory transcription factor in myeloid cells responsible for the production of IL-1b, iNOS, and other cytokines implicated in atherosclerosis. It is believed that HDL inhibits NFκB activation by blocking a sphingosine kinase signaling pathway upstream of NF-κB[413, 414]. Monocytes from patients with liver cirrhosis are inherently pro-inflammatory; however, recombinant HDL was shown to block NF-κB activation in these cells and prevent their pro-inflammatory phenotype. The authors of this study suggest that the ability of HDL to neutralize LPS attenuates NF-κB activation[415]. ApoA1 plays a central role in HDL-dependent LPS neutralization as distinct amino acid substitutions in ApoA1 attenuates the LPS-neutralizing capacity of HDL[416].

In addition to neutralizing endotoxin, HDL also has direct and indirect anti-inflammatory activity through its major role as an acceptor of macrophage cholesterol. Indirectly, HDL-mediated cholesterol efflux prevents inflammatory cytotoxicity in macrophages. Indeed, macrophage-specific deletion of ABCA1, which results in the accumulation of free cholesterol, increases cytokines production in response to LPS stimulation[417]. Moreover, the HDL-dependent efflux of oxidized lipids – many of which promote vascular inflammation[418] – is consistent with the observation that the ability of HDL to inhibit monocyte chemotaxis correlates strongly with the HDL efflux capacity[397]. A direct effect of HDL on inflammatory signaling has also been proposed: The transduction of inflammatory signals across the cellular membrane requires the formation of lipid rafts containing high concentrations of cholesterol and sphingolipids. Cholesterol

depletion from these microdomains affects subsequent signaling processes[419], and apoA1 has been shown to disrupt lipid rafts by efficiently depletes cholesterol from macrophage membranes[420]. Thus, the direct effect of HDL and ApoA1 on lipid rafts may explain some of HDL's reported anti-inflammatory activity in immune cells.

One of the initiating steps in atherogenesis is the activation of the vascular endothelium resulting in an increase in the expression of adhesion molecules (e.g. E-selectin, VCAM-1, ICAM-1) and secretion of chemotactic factors. HDL can inhibit cytokine-induced expression of adhesion molecules on human endothelial cells and thereby reduce the adhesion and migration of monocytes[421, 422]. Consistent with these *in vitro* findings, administration of recombinant HDL or apoA1 reduces adhesion molecule expression and monocyte infiltration in mice[423].

In sum, HDL exerts multiple effects that may lower the activation threshold of immune cells within arterial lesions (i.e. decreasing the intracellular levels of pro-inflammatory oxidized lipids, attenuation of NF-κb signaling, etc.) thereby reducing the ability of these cells to responds to inflammatory cytokines and microbial stimuli (LPS). This multi-factorial function of HDL is important during times of acute infection, such as in bacteremia, as well as under conditions of chronic inflammation. The inflammatory state, however, can also exert powerful effects on HDL composition and function; these effects will be discussed in a following section. For now, the therapeutic benefits of targeting HDL levels for atherosclerosis will be explored.

Is raising HDL beneficial?

Transgenic and adenoviral methods have been employed to increase HDL production in mouse models of cardiovascular disease[424-426]. These studies all resulted in

decreased cellular cholesterol and atherosclerotic plaque cellularity and afforded atheroprotection under both chow and western diet conditions[427]. Yet, as described above, the human trials that have raised plasma HDL-C have consistently failed to show clinical benefit for CVD[364, 365, 428]. A major difficulty in interpreting the result of the available clinical studies, however, is that none of them were able to establish whether the functional activity of HDL was similarly increased. Major efforts, therefore, are currently underway to find markers of HDL function that can be used clinically to measure the benefits of various HDL-altering strategies. A marker of HDL function is highly desirable since most patients only present to the clinic once there is already significant disease burden. There is now strong supporting data that increasing the number of functional HDL particles induces plaque remodeling[429-431], particularly in the content and inflammatory phenotype of lesion macrophages. Thus, strategies that raise the amount of functional HDL may not only protect against the progression of atherosclerosis, but also promote its regression – an important point considering the state in which most patients first present to the clinic.

HDL raising strategies

Niacin Niacin, the first anti-dyslipidemia agent identified[432], remains the most potent drug for increasing HDL-C levels (by 15% to 30%), however it has limited use due to its side-effect profile[432]. Niacin appears to increase HDL levels by decreasing the hepatic uptake of apoA1, thereby attenuating the catabolism of HDL[433]. The AIM-HIGH trial was the first large-scale outcomes-based study to evaluate the impact of adding extended release niacin to statin therapy in patients with existing coronary artery disease[368]. The study was designed to test whether increasing HDL-C in patients with controlled LDL-C

levels would reduce the risk of recurrent cardiovascular events. The trial was halted prematurely because interim analyses showed no clinical benefit for patients receiving Niacin. The "nail in the coffin" for niacin might have come from the recent release of the findings from the HPS-2-Thrive study. This secondary prevention study using extended-release niacin with statin alone or in combination with Ezetimibe also failed to show clinical benefit for niacin[365].

CETP inhibitors Cholesterol ester transfer protein (CETP) mediates the bidirectional transfer of lipids between triglyceride rich lipoproteins and HDL. Rodents do not express CETP and are relatively resistance to high-fat diet induced atherosclerosis. Transgenic exogenous CETP expression in athero-susceptible mice (*ApoE*$^{-/-}$ or *Ldlr*$^{-/-}$), however, results in increased atherosclerosis[434]. Plasma CETP mass and activity are elevated in CVD patients and those at increased risk, resulting in decreased HDL and increased triglycerides. Preliminary studies have also revealed a positive correlation between the carotid intima media thickness (early quantification of athero burden) and CETP concentration[435, 436]. Three single nucleotide polymorphisms in the CETP gene are associated with decreased CETP activity and elevated HDL-C levels in carriers and inversely related to CVD risk[325, 437]. Together, the correlation between CETP and CVD in humans led to the idea that CETP inhibition would be a reasonable HDL-C based therapeutic target. In rabbit models, CETP inhibitors decreased CETP activity by more than 70%, resulting in a 35% increase in HDL-C and reduction in atherosclerosis[438].

Statins HMG-CoA reductase inhibitors (statins) have pleiotrophic effects and in addition to lowering LDL-C they have been shown to modestly raise HDL-C level as well. The HDL-C raising effects have been attributed in part to inhibition of rho-signaling pathways

and concurrent activation of PPARα[439]. Moreover, statins also reduce plasma CETP activity, which could also contribute to increased HDL-C levels. Interestingly, statin treatment has been shown to confer the strongest cardioprotective effects on patients with lower baseline HDL-C[101]. Thus, although their HDL-raising effects are modest, statins reduce CV risk in patients with low HDL-C.

Fibrates Fibrates are ligands for PPARα and in addition to lowering LDL-C and triglycerides they have been shown to increase the expression of ApoAI[440] and modestly raise HDL-C levels[441, 442]. Treatment with fibrates significantly reduces coronary events in patients and similar to statins, the cardioprotective effects are stronger in individuals with lower baseline HDL-C levels[441]. In all five clinical trials to date, fibrates appear to have the greatest benefit for patients with atherogenic dyslipidemia (low HDL-C, high triglycerides, and prevalence of small LDL). Indeed, a recent meta-analysis of dyslipidemic subgroups from the fibrate trials showed a 35% relative risk reduction in cardiovascular events compared to a non-significant 6% reduction in those without dyslipidemia[443]. Whether fibrates confer cardioprotection by raising HDL mass or by altering other parameters of atherogenic dyslipidemia remains to be determined. The severe side effects associated with fibrates, especially in combination with statins (e.g. rhabdomyolysis), however, will likely limit their clinical use for the treatment of CVD.

Strategies to directly increase ApoA1 levels Human ApoA1 transgenic mice have elevated levels of HDL and are protected against atherosclerosis[444]; providing evidence that ApoA1 overexpression can increase HDL-C and protect against atherosclerosis. A major question, however, is whether the rate of ApoA1 production is a major determinant of HDL quantity and quality in humans. The ApoA1 gene is regulated primarily by *cis*-

acting elements and partially at the post-translational level by factors that increase mRNA stability[445, 446]. Dietary fat, alcohol, estrogen, androgens, thyroid hormones, retinoids, glucocorticoids, fibrates, niacin, and HMG-CoA reductase inhibitors are some of the many nutritional, hormonal and pharmacological factors known to influence induction of the ApoA1 gene[446]. Changes in diet have been shown to affect HDL levels[52, 447, 448]. Such interventions, such as a switch from high-carbohydrate to a high-fat diet appear to exert their major effect on by altering the production rates of apoA1[449]. In addition to uncovering factors that increase the biosynthesis of ApoA1, measures that decrease its catabolism are also being explored. In fact, *in vivo* studies of HDL metabolism indicate that over a wide range of body weights and plasma TG levels the rate of ApoA1 clearance rather than its rate of production is the most important determinant of HDL-C and ApoA1 variability in humans. Within phenotypically similar groups (narrow range of body weights, triglyceride levels, etc.), however, the production rate of apoA1 is an important determinant of the variability of plasma HDL levels[450-452].

Augmentation of lipid-poor ApoA1 represents the most validated HDL-related therapeutic approach to raise HDL levels and/or function. Infusions of lipid-poor apoA1-phospholipid complexes, often referred to as recombinant HDL (rHDL), have been extensively studied in animals and in pre-clinical studies in humans. These preliminary studies have demonstrated that the administration of apoA1 is athero-protective and promotes regression of disease[453-455]. Consistent with these findings, apoA1 has been shown to not only promote reverse cholesterol transport[393], but also to inhibit vascular inflammation[456], the expression of endothelial adhesion molecules[457], and phospholipid oxidation[458]. All of these effects could contribute to the anti-atherogenic potential of

rHDL administration, and early clinical studies in humans have shown that rHDL infusion is well tolerated and decreases coronary atherosclerosis to the similar extent as long-term statin use[459]. An alternative strategy to augment HDL levels involves upregulation of endogenous apoA1 synthesis. Recently a small molecular compound (RVX-208) developed by Resverlogix Corporation was shown to selectively upregulate apoA1 synthesis in hepatocytes[460]. The recent release of their phase II clinical trials data demonstrates that treatment with RVX-208 leads to a significant reduction in major adverse cardiac events. Furthermore, the clinical benefit of RVX-208 was greater in patients with high levels of inflammation (CRP > 2.0mg/dL)[461].

Conclusion The recent failures of the niacin and CETP inhibitor trials have raised serious doubts about the relevance of raising HDL-C as an atherosclerotic therapy. While niacin and CETP inhibitors raise HDL-C, they do not increase the number of HDL particles. Recent findings indicate that HDL particle number is a better indicator of HDL function than HDL-C values[412]; thus, this may be part of the explanation for why niacin and CETP inhibitors have not shown clinical benefit. Additionally, it is quite possible that therapies aimed at raising HDL levels fail because they do not mitigate the effects of inflammation (PL depletion, TG enrichment, and increases in pro-inflammatory proteins) on HDL particle function. HDL-raising therapies either increase HDL production or reduce HDL catabolism. Increasing HDL in the presence of chronic inflammation such as seen in atherosclerosis may have the paradoxical effect of merely generating more dysfunctional or even pro-inflammatory forms of HDL. Presumably, decreased catabolism of dysfunction HDL also is unlikely to be beneficial for CVD treatment. Being able to

measure HDL function, therefore, will be an invaluable tool as the field explores the therapeutic potential of HDL-targeted therapies.

Effects of inflammation on HDL function

The risk of atherosclerosis is increased in many chronic inflammatory disorders including infection with *Helicobacter pylori*, chronic bronchitis, chronic kidney disease, rheumatoid arthritis and systemic lupus erythematous (SLE)[462-464]. The first observation that inflammation alters HDL form and function arose from studies investigating HDL composition during the acute phase response or influenza A infection[410, 465]. These studies demonstrated that Inflammation promotes the incorporation of inflammatory cargo, such as acute phase proteins, into the HDL particle. Importantly, these "pro-inflammatory" HDL particles are less able to promote macrophage cholesterol efflux[24], protect LDL against oxidation, or to inhibit the increased expression of adhesion molecules associated with inflammation[410]. The hypothesis that certain diseases renders HDL dysfunctional was further supported by the observation that despite high HDL-cholesterol levels, HDL from patients with CVD had less anti-oxidative activity[466]. This finding led to a series of studies which found that cohorts of patients with systemic lupus erythematosus (SLE), end-stage renal disease (ESRD) and metabolic syndrome had HDL with decreased anti-oxidant activity as well[467]. These results intrigued researchers and have led to a massive effort to characterize dysfunction HDL and determine precisely how inflammation alters HDL composition and function.

Inflammation alters HDL proteins Recent proteomic studies have analyzed HDL from patients with high cardiovascular risk. Interestingly, there are multiple changes in the

protein composition of HDL particles isolated from patients with coronary artery disease[468, 469], chronic kidney disease[470, 471], rheumatoid arthritis[472], and psoriasis[473]. The presence of any one of these inflammatory diseases is associated with HDL enriched in the acute phase protein serum amyloid A (SAA) and complement component 3 (C3). C3 is a modulator of the innate immune system[474] and has been implicated in contributing to vascular disease[475]. HDL isolated from these patients also displayed decreased apoA1 levels, consistent with the finding that SAA can replace apoA1 from HDL under inflammatory conditions[476, 477]. Weichhart et al. recently demonstrated that HDL from patients with chronic kidney disease stimulates cytokine production and adhesion molecule expression on monocytes and dendritic cells. Using shotgun proteomics they identified a uremic HDL protein signature, and of all the associated proteins only SAA was found to be responsible for the pro-inflammatory effects[471]. These findings are consistent with recent evidence that SAA stimulates innate immune responses[478] and that SAA is enriched in patients with acute coronary syndrome[469].

Other proteins found to be increased on inflammatory HDL particles include ApoCII, ApoCIII and ApoAIV[470]. Previous studies have shown that ApoCIII is inhibitory against lipoprotein lipase and hepatic lipase[479]. As described above, alterations in the activity of these lipases can impact HDL levels and composition. ApoCIII is also pro-inflammatory mediator and can directly activate monocytes through TLR2 and NFκB signaling[480]. This effect of ApoCIII promotes atherosclerosis[480], suggesting a novel mechanism by which inflammatory HDL particles may contribute to atherogenesis.

Interestingly, even conditions characterized by low-grade inflammation such as psoriasis have been associated with changes in HDL composition and function. HDL

isolated from psoriatic patients with relatively modest inflammation (median CRP of 2.7 mg/dL, within the accepted levels of 1-3 mg/dL) had decreased ApoA1levels and increased acute phase proteins, including SAA and prothrombin[473].

The proteomic analysis of HDL is still in its infancy; yet, the data so far suggests that diseases with increased CVD risk, such as rheumatoid arthritis, end-stage kidney disease, and diabetes have a characteristic HDL proteome harboring various pro-inflammatory proteins. These initial studies, however, need further validation before the HDL proteome can be used as a biomarker for disease. Moreover, how these changes in HDL composition affect HDL function(s) and the progression of atherosclerosis is still not well understood.

Inflammation alters HDL lipids In contrast to HDL proteomics, less is understood about the lipid composition of HDL and how the HDL lipidome changes in association with various inflammatory diseases. Compared to apoB-containing lipoprotein particles, HDL is enriched in phophatidylcholine, lysophosphatidylcholine, and phosphatidylethanolamine. Consistently associated with CVD and other inflammatory disorders is the significant reduction in HDL phospholipids and total cholesterol content[470, 473, 481, 482]. Interestingly, studies using mice and rats expressing human APOA1 indicate that the prime component of HDL that modulates cholesterol efflux is HDL phospholipid[483, 484]. 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 triglycerides[485]. Consistent with the changes in HDL phospholipids observed in inflammatory diseases, HDL isolated from patients with chronic kidney disease, rheumatoid arthritis and

psoriasis were shown to be less effective at promoting macrophage efflux[470, 473, 486]. Together these findings suggest that the lipid composition of HDL might play an important role in influencing HDL function.

Obesity and HDL dysfunction

The incidence of obesity has doubled in the United States since 1960, with more than one third of the adult population currently obese[487]. The incidence of obesity among children has also risen from 6% to 19% over the past 25 years[488]. Obesity has been associated with several conditions including type 2 diabetes, hypertension, hypercholesterolemia, hypertriglyceridemia and nonalcoholic fatty liver disease. As a result, a conservative estimate puts the medical costs related to obesity at approximately $150 billion per year in the US[489].

One of the first studies to demonstrate an association between obesity and CVD risk was the large, cross-sectional multinational Lipid Research Clinics Program Prevalence Study, which found a significant inverse correlation between the Quetelet index of body mass (measured as height divided by weight squared) and total HDL-C in both men and women[490]. The association remained significant when the authors controlled for confounding variables that affect HDL-C levels. In another large-scale study, plasma HDL-C and ApoA1 levels both significantly declined in a linear fashion with increasing BMI[491]. An increase in BMI was also more strongly related to reduced HDL levels than other CV risk factors including LDL-C levels[491]. The Quetelet index and BMI are indirect indices of obesity and more direct measurements of adiposity have uncovered a strong correlation specifically between intra-abdominal fat and CV risk including low HDL-C levels[492, 493]. Although there is strong correlation between obesity

and CVD[203], no epidemiologic or clinical study have yet established a causal link between the low plasma HDL-C concentrations observed in the obese state and an increased risk for CVD. Other lipoprotein abnormalities and metabolic changes also occur in the obese state and likely contribute to the increased CVD risk associated with obesity. These changes, however, are less consistent than the observed effect of obesity on HDL-C levels.

HDL lowering in obesity In addition to lower HDL levels, obesity has also been shown to adversely affect the distribution of HDL subfractions and to alter the composition of HDL particles. Several studies have shown that HDL from obese subjects has reduced cholesterol and protein contents; moreover, the concentration of atheroprotective HDL_2 ("small" HDL; density between 1.063 and 1.125 g/mL) particles specifically is decreased[493-495]. Both direct and indirect mechanisms have been proposed to account for the reduced HDL_2 cholesterol levels observed in the obese state. First, other metabolic abnormalities that are known to influence plasma lipid levels are linked to obesity. Hypertriglyceridemia, in particular, is frequently associated with lower HDL levels and increased HDL catabolism in obese subjects[496]. In hypertriglyceridemia CETP mediates a greater net transfer of triglycerides from apoB-containing lipoproteins to HDL than normal resulting in TG-rich, cholesterol-depleted HDL_2 particles[497]. Triglyceride-rich, large HDL_2 are the preferred substrate for hepatic lipase, which hydrolyzes HDL and promotes its uptake by the liver[498, 499]. Since obese individuals have been found to have increased CETP mass and activity[500], triglyceride enrichment of HDL_2 by CETP may explain, at least in part, why HDL_2 levels are reduced in hypertriglyceridemic obese subjects. PLTP also plays a dominant role in HDL remodeling and have been shown in

vitro to catabolize HDL_2 to small pre-β HDL, activity which is enhanced by enrichment of HDL with triglycerides[501]. Evidence that PLTP may also influence the changes in HDL levels observed in obesity is provided by studies indicating that PLTP activity is increased in obesity and is positively related to BMI[502] and that weight loss results in a significant decrease in PLTP activity and concomitantly, an increase in HDL_2 particle size[503].

There is evidence as well that obesity can directly reduce HDL levels. Clinical studies indicate that weight loss, and specifically loss of adipose tissue, induces a rise in HDL-C in obese patients[504, 505]. These clinical studies are consistent with *in vitro* data that adipose can specifically bind and mediate the uptake of HDL[506, 507]. Moreover, the uptake of HDL_2 by adipose appears to be tissue specific with abdominal adipocytes mediating a greater uptake[506, 507]. In addition, HDL_2 uptake by adipocytes is dependent on fat cell size, with larger cells taking up more HDL_2 particles[507]. These findings are consistent with reports that abdominal adiposity is associated with CV risk[492]. Analogous to HDL_2 levels, apoA1 levels are also low in obese patients[508]. To study the factors responsible for reduced ApoA1 in the obese state several studies used radioisotopes to trace apoA1 in normolipidemic, non-smoking individuals[450, 509]. Compared to controls, obese subjects had a 30% reduction in the residence time of apoA1 in circulation[509]. Conversely, the rate of apoA1 appearance into the plasma was no different between obese and control subjects, indicating that obesity directly enhances the clearance of apoA1 (i.e. HDL) in plasma[450]. These findings were later confirmed by a studying in which the level of intra-abdominal fat strongly correlated with the clearance rate of apoA1[510].

1.4 Liver X Receptors

The liver X receptors (LXRs) are nuclear hormone receptors that play a central role in cholesterol homeostasis and lipid metabolism. LXRα (NR1H3) and LXRβ (NR1H2) were cloned twenty years ago based on sequence homology with other receptors and were so named because of their isolation from a human liver cDNA library. LXRs were originally assigned to the orphan family of nuclear receptors because their natural ligands were unknown [511, 512]. Proteins in this group are defined by the presence of a ~70 amino acid stretch encoding a highly conserved DNA-binding domain that targets the receptors to specific DNA binding elements in the promoter region of target genes. The c-terminus of the receptor contains a larger, less-conserved ligand-binding domain responsible for hormone binding, dimerization, and ligand-dependent receptor activation[513]. Upon ligand-binding, nuclear receptors undergo a conformational change, thereby releasing associated corepressor proteins in exchange for coactivators that promote gene transcription[514, 515].

The DNA and ligand binding domains of LXRα and LXRβ are highly homologous (>75%); yet, they are encoded by two distinct genes (*LXRα* on chromosome 11p11.2 and *LXRβ* on chromosome 19q13.3) and display differences in expression patterns. LXRβ is ubiquitously expressed while LXRα is highly expressed in the liver and at lower levels in the intestine, adipose, adrenal, macrophage, kidney, and lung [511, 512, 516] The LXRs form permissive, obligate heterodimers with the retinoid X receptor (RXR), a nuclear receptor bound and activated by 9-cis retinoic acid. The RXR/LXR heterodimer can be activated by either receptor's ligands[517], but the activity of the complex depends solely on LXR to elicit the transcriptional response[518]. The RXR/LXR heterodimer binds preferentially to DNA sequences consisting of two conserved

hexanucleotides separated by 4 bases (a DR-4 motif referred to as an LXRE)[512, 519]. Such a motif was then quickly identified within the promoter of the rat *Cyp7a1* gene, which encodes for the rate-liming enzyme, CYP7A, in the bile-acid synthesis pathway. Activation of a *Cyp7a1* reporter construction *in vitro* was determined to be RXR/LXR-dependent [520].

Discovery of LXR ligands Cell based assay using a *Cyp7a1*-reporter construct was then used to screen tissue extracts for LXR ligands and it was found that oxysterols could activate the RXR/LXR heterodimer. Oxysterols are cholesterol metabolites derived from enzymatic and non-enzymatic oxidation. Oxidation makes these molecules more hydrophilic and reduces their half-life. With few exceptions (e.g. atheromas), oxysterols are present in trace amounts and are important intermediates in the elimination of cholesterol from liver and extrahepatic tissues. Subsequent screenings revealed that the most potent LXR ligands are 22(R)-hydroxycholesterol (22-HC), 24(S)-hydroxycholesterol (24-HC), and 24(2),25-epoxycholesterol (24,25-EC)[517, 520, 521]. These ligands bind LXRα and LXRβ similarly and within the range of physiological concentrations [512, 520]. Desmosterol, an intermediate in the cholesterol biosynthesis pathway, is also an effective LXR activator[522] Although the binding affinity of desmosterol for LXRs is about one fifth that of the most potent oxysterol ligand, 24,25-EC[522], desmosterol is enriched ~20 fold in foam cells and is the dominant LXR ligand in these cells *in vivo*[523]. Two non-steroidal synthetic LXR agonists, T0901317 and GW3965 are commonly used in experimental studies. T0901317 activates LXRα and LXRβ with an EC_{50} of 20nM[524] and in contrast to GW3965 has sustained activity in the liver (Breevoort, unpublished data). T0901317 also activates the farnesoid X receptor (FXR) [525] and the pregnane X receptor (PXR)[526]. These two receptors influence lipid and glucose metabolism and hepatic lipid accumulation,

respectively. Treatment with T0901317 induces a dramatic increase in hepatic lipogenesis[527, 528]. Some have speculated that T0901317 activation of FXR and PXR in the liver may contribute to this difference, however, in the absence of liver LXRα expression, T0901317 does not increase plasma TGs or hepatic cholesterol accumulation[529]; therefore, these effects of T0901317 are dependent on LXRα activity in the liver.

Alternative regulation of LXR expression and activity Apart from activation by ligands, LXRs can also be regulated by other mechanism. For instance, the *Lxrα* gene is auto-regulated in human macrophages [530] and its expression can be additionally modified by other factors including thyroid hormone and other cytokines[531-533]. The activity of LXRs can also be modulated through post-translational modifications including phosphorylation, acetylation, SUMOylation, and O-GlcNACylation[1, 534-540]. The phosphorylation of LXRs appears to drive gene- and cell-type specific regulation of LXR-agonist mediated gene expression[534-538]. SIRT1 promotes LXR dependent gene expression by relieving the repressive acetylation of the receptor[539]; SUMOylation of LXRs plays a key role in transrepression[1] and O-GlcNACylation has been reported as a mechanism through which LXRs can act as glucose sensors[540].

Identifying the role for LXRs in cholesterol homeostasis. The tissue expression patterns of LXRs, the identification of oxysterol ligands, and the characterization of the LXRE in the *Cyp7a1* promoter suggested that LXRs influence bile acid synthesis. Wildtype mice challenged with a 2% cholesterol diet have an increase in CYP7A1 mRNA levels and consequently bile acid pool size and fecal bile acid excretion increases[541]. These effects, however, were absent in the Lxrα knockout mice and as a consequence these animals

accumulate increased hepatic cholesterol[541]. Although Lxrβ is also expressed in the liver, it does not seem to compensate for the loss of Lxα; Indeed, the LXRβ knockout mouse does not exhibit cholesterol-induced changes in bile acid metabolism[516].

Dietary cholesterol-induced up-regulation of CYP7A1 expression varies widely among species. Rat and mouse show pronounced up-regulation, however, humans show minimal change[542]. There are two sequence differences in the LXRE of the human *Cyp7a1* promoter that significantly decreases the DNA binding of LXR *in vitro*[543].

Further analysis of gene expression in $Lxr\alpha^{-/-}Lxr\beta^{-/-}$ cells uncovered diminished levels of SREBP-1 and stearoyl-CoA desaturase mRNA[541] indicating that LXRs play a significant role in regulating fatty acid metabolism. Together, these early studies implicated a clear role of LXRs in whole body cholesterol homeostasis and through additional studies it has become clear that LXR exerts this role through its ability to regulate reverse cholesterol transport, inhibit intestinal cholesterol absorption, and promote hepatic lipogenesis. In addition, LXRs indirectly modulate the expression of certain genes by either enhancing or repressing the actions of other transcription factors[544-547]. Such indirect regulation of additional pathways may also contribute to the physiological responses to LXR agonists observed in animal studies.

LXRs and atherosclerosis

LXR agonists are atheroprotective LXRs are involved in many steps of the atherogenic pathway and by regulating important genes involved in lipid homeostasis and inflammation, LXRs have many reported anti-atherogenic properties. LXR agonists have unequivocally now been shown to be anti-atherogenic in various animal models of atherosclerosis. Initial studies demonstrated that the synthetic LXR agonist, GW3965

inhibited lesion development in both *apoE*$^{-/-}$ and *Ldlr*$^{-/-}$ mice[548]. Additional studies have confirmed these initial observations using various LXR agonists and different mouse models[529, 549-555]. Importantly, the beneficial effects of LXR activation is not sex specific[548, 554, 555]. In some studies, the reduction in atherosclerosis following LXR agonist treatment was associated with reduced total cholesterol and/or elevated HDL cholesterol[548, 549, 552, 554], each associated with reduced cardiovascular risk in humans[362]. Yet, raising HDL-C and/or lowering LDL-C is not required for the athero-protective effects of LXR agonists; we and others have observed a reduction of atherosclerosis without a change in plasma lipids[529] In addition, several studies have shown reductions in atherosclerosis in the absence of effects on SREBP1c and hepatic lipogenesis whereas others report a reduction in atherosclerosis despite increased TG levels[549, 553, 554]. Together, these observations suggest that the beneficial effects of LXR agonists are independent of systemic lipid metabolism and perhaps act more directly in the vessel wall. Indeed, the athero-protective properties of LXR agonists are lost in the absence of hematopoietic LXR expression[549] yet maintained in liver specific *Lxrα*$^{-/-}$ */Ldlr*$^{-/-}$ mice[529]. It is important to note as well that not only have these studies demonstrated that LXR agonists attenuate lesion development, but also that LXR agonists can promote the modulation of the plaque itself[529, 549] and stimulate plaque regression[555, 556]. This is relevant to therapeutic development of LXR agonists since patients usually already have established lesions before presenting for treatment for cardiovascular disease.

Which LXR subtype is responsible for the anti-atherogenic effects? Researchers have used gene deletion studies in attempts to define the anti-atherogenic activities of the LXR subtypes. Deletion of *Lxrα* or *Lxrβ* alone has no apparent phenotype in animals on chow

diet; however, deletion of both subtypes together lowers serum triglycerides and high-density lipoprotein cholesterol and increases LDL cholesterol[557]. In the absence of any atherogenic signal, *Lxrα*$^{-/-}$ *β*$^{-/-}$ mice have increased aortic foam cell accumulation after 18 months of normal-chow diet feeding[557]. The increase in atherosclerosis in the *Lxrα*$^{-/-}$ *β*$^{-/-}$ mice is predominately mediated by LXRα, as the *Lxrα*$^{-/-}$ *apoE*$^{-/-}$ mouse accumulated increased cholesterol in the liver and had increased atherosclerosis[558]. These findings are consistent with earlier reports and add that in the setting of hypercholesterolemia, LXRβ is not compensatory[558]. Yet LXR agonist treatment reduces cholesterol accumulation and atherosclerosis in *LXRα*$^{-/-}$*/ apoE* and there is no increase in plasma triglycerides associated with LXR activation[558] indicating that LXRα mediates this effect. Indeed, Similar studies in *Ldlr*$^{-/-}$ mice showed that deletion of LXRα but not LXRβ is associated with increased atherosclerosis[559], supporting the dominant role for LXRα in providing athero-protection. Experiments that selectively rescued LXRα function in bone marrow cells or extra-hematopoietic cells suggested that LXRα activity is required in both the macrophages as well as another non-hematopoietic site to limit diet-induced CVD[559]. Moreover, overexpression of LXRα in *Ldlr*$^{-/-}$ mice reduces atherosclerosis[560]. Together these findings point to LXRα as the subtype responsible for the majority of the LXR dependent anti-atherogenic activity. The potential anti-atherogenic activities of LXR agonists and the role of LXRs in atherosclerosis can be found in Tables 1.1 and 1.2, respectively.

LXR and reverse cholesterol transport

Uptake of oxLDL by macrophages in the vessel wall is a critical initiating step in atherosclerosis. RCT, as described previously, is the primary mechanism by which

cholesterol is removed from peripheral cells including lipid loaded foam cells. Importantly, Naik et al. pioneered a model to measure RCT *in vivo* and then demonstrated that LXR agonist treatment could increase RCT[561]. We now know that most, if not all, steps of the RCT pathway are governed by LXR (Figure 1.2). First, in response to ligand or increasing intracellular cholesterol levels, LXR promotes the expression of ABCA1 and ABCG1in macrophages allowing for increased cholesterol efflux to acceptor particles[562](Figure 1.2). In addition, activators of peroxisome proliferator-activated receptor (PPAR)-α and –γ, erythropoietin, and atorvastatin are known to stimulate macrophage cholesterol efflux in a LXR-dependent manner[563-566]. In addition to influencing cholesterol efflux, LXRs also play a role in inhibiting macrophage cholesterol uptake[567, 568]. This effect is mediated by LXR dependent reductions in macrophage pinocytotic vesicles[567] and increases in Idol expression, resulting in ubiquitination of LDLR and its subsequent degradation[568]. As described above, important roles for macrophage LXRs have been uncovered; Transplantation of LXRα and LXRβ deficient bone marrow into *apoE*$^{-/-}$ and *Ldlr*$^{-/-}$ recipient mice strongly increased lesion development [569]. Moreover, isolated macrophages from *Lxra*$^{-/-}$ *Lxrb*$^{-/-}$ mice accumulate increased cholesterol *in vitro*[569]. Furthermore, overexpression of LXRα in macrophages specifically reduces atherosclerosis without altering plasma lipid levels in hypercholesterolemic *Ldlr*$^{-/-}$ mice[560]. Together, these findings indicate that LXR, and particularly LXRα, activity in the macrophage is critical for limiting atherosclerosis. Also, LXR agonists are ineffective against lesion development in *Ldlr*$^{-/-}$ mice with LXR deficient bone marrow, suggesting that most if not all of the anti-atherogenic effect are derived from activating LXRs in hematopoietic cells[549]. In addition to mediating

cholesterol uptake and efflux, macrophage LXR also represses inflammation[570], directs cell egress[556], and limits endoplasmic reticulum (ER) stress[571], activities which may all contribute to varying degrees to the anti-atherogenic properties of macrophage LXR.

LXR regulates cholesterol excretion and absorption The increased cholesterol effluxed from macrophages is transported by HDL to the liver where LXR directs its metabolism and excretion (Figure 1.2). The importance of LXRα activity in the liver is evident as mice lacking liver LXRα expression accumulate hepatic cholesterol when fed a high cholesterol diet[529, 572]. In mice, this defective cholesterol clearance has been shown to be related to the reduced expression of *Cyp7a1*[541]. As *Cyp7a1* is only minimally regulated by LXR in humans[542], the contribution of this pathway in humans is probably not significant. Perhaps more important in humans is the regulation by LXR of the two half transporters ABCG5 and ABCG8 that act as a dimer to facilitate cholesterol transport[573-575] (Figure 1.2). ABCG5/G8 are primarily expressed in the liver and in the intestine where they promote cholesterol excretion (liver) directly into the bile and limit cholesterol absorption (intestine). In contrast to wild-type animals, LXR agonists fail to stimulate biliary cholesterol excretion and reduce cholesterol absorption in ABCG5/G8 double knockout animals[573], indicating that these transporters play an important role in LXR mediated cholesterol homeostasis. In addition, LXR agonists decrease the expression of NPC1L1, a protein critical for intestinal cholesterol absorption, in the apical membrane of enterocytes in humans and mice[576], which is another mechanism leading to LXR dependent reductions in cholesterol absorption.

However, an increase in fecal cholesterol excretion following LXR agonist treatment is not entirely dependent on biliary cholesterol excretion. GW3965 treatment,

although not increasing biliary cholesterol content raises fecal cholesterol levels in *Mdr2*$^{-/-}$ mice, which are unable to secrete cholesterol into bile[577]. These findings suggest that LXR activity in the intestine may provide an alternative pathway for cholesterol excretion. This pathway, termed Trans-intestinal cholesterol excretion (TICE), proposes that HDL directly transfers cholesterol from peripheral cells to the intestine, where it is removed to the gut lumen and excreted into the feces.

LXR increases HDL-C levels The liver and the intestines are the two major sites of HDL biosynthesis. The liver generates lipid-poor ApoA1 and nascent HDL while the intestine plays a key role in HDL maturation through ABCA1-dependent lipidation of nascent particles[372]. LXR agonists increase ABCA1 expression in human enterocytes *in vitro* resulting in an increase in apoA-1 mediated cholesterol efflux[578]. Brunham et al. preformed studies with tissue-specific ABCA1 deletions and found that Intestine-specific deletion of ABCA1 reduces serum HDL-C by 30% and that LXR agonists raise HDL levels in wild-type mice as well as in hepatic-specific ABCA1 deficient animals, but fail to do so in intestine-specific ABCA1 knockouts[579, 580]. In addition, the effect of T0901317 on plasma HDL levels is lost in *Npc1l1*$^{-/-}$ mice that are deficient for intestinal cholesterol absorption, indicating that gut cholesterol levels are important for HDL formation in response to LXR agonists[581]. Ezetimibe is a potent inhibitor of intestine NPC1L1 effectively lowering plasma LDL-C[582]; yet, targeting intestinal cholesterol absorption may also have the dual effect of raising HDL levels through an LXR mediated pathway.

LXRs regulate lipid metabolism

Schultz et al.[524] and Repa et al.[583] simultaneously showed that LXR agonists raise hepatic and plasma triglyceride levels in mice and hamsters. While the effect on plasma triglycerides was transient, hepatic triglyceride levels persisted and led to liver steatosis and dysfunction. In addition, Schultz et al. found that plasma triglycerides were about 4-fold lower in *Lxrα*$^{-/-}$ *Lxrβ*$^{-/-}$ mice compared to wild-type controls. The LXR agonist dependent increase in hepatic and plasma triglycerides observed by Schultz et al. is a result of LXR-stimulated lipogenesis in hepatocytes. This effect is mediated by increased expression of SREBP-1c, a transcription factor that acts a master regulator of fatty acid synthesis. SREBP-1c binds to sterol response elements (SRE) within the promoter region of target genes encoding lipogenic enzymes [583]. There are two LXREs within the SREBP-1c gene promoter, and LXR as well as RXR agonists increase its transcriptional activity [584]. In addition, LXR directly regulates the expression of several lipogenic enzymes including acetyl-CoA carboxylase (ACC)[585], fatty acid synthase (FAS)[586], and stearoyl-CoA desaturase-1 (SCD-1)[587]. LXR agonists increase SREBP-1c, ACC, FAS, and SCD-1 gene expression in the liver of wild-type and LXRβ$^{-/-}$ mice, but not in *Lxrα*$^{-/-}$ animals [524]. In addition, basal levels of these lipogenic genes are reduced in *Lxrα*$^{-/-}$ mice. Together, these findings indicate that LXRα is the major isoform responsible for the lipogenic effect of LXR agonists. Apart from the direct (SREBP-1c independent) and indirect (SREBP-1c dependent) effects of LXR agonists on lipogenic genes, other mechanisms for LXR-stimulated lipogenesis have been described. For instance, LXREs have been found in the promoter region of the carbohydrate response element binding protein (ChREBP) gene[588]. ChREBP is a glucose-sensitive transcription factor and upon sensing increased glucoses levels it will stimulate the expression of lipogenic genes. LXR

agonists stimulate the expression of ChREBP in the liver in both in vitro and in vivo models, and the agonist-dependent upregulation of FAS, ACC, and SCD-1 are blocked in ChREBP deficient mice[588].

The physiological result of increased hepatic LXRα activity is the production of triglyceride-rich, large VLDL particles [527]. Although the number of VLDL particles does not change, their diameter increases due to the greater number of triglyceride molecules per particle[589]. LXR agonist treated animals show only a transient rise in plasma triglycerides because VLDL metabolism is also increased, presumably through upregulation of lipoprotein lipase, which is a direct LXR target gene[590].

LXRs and lipoprotein particles

LXR regulates apolipoproteins Apoliprotein E (apoE) is an alternative extracellular cholesterol acceptor found in many classes of lipoproteins and is involved in LXR-mediated cholesterol efflux. ApoE was the found to be under the regulation of LXR both *in vitro* and *in vivo*[591]. In addition to apoE, LXR agonists also stimulate the expression of other apolipoproteins in the same gene cluster as apoE including apoC-I, apoC-II, and apoC-IV. The functions of these proteins are still being worked out, however, they are known to influence lipoprotein metabolism[463] and can act as cholesterol acceptors[592].

In humans, ApoA-IV is synthesized exclusively in the intestine; whereas in rodents ApoA-IV is generated in the intestines and, to a lesser extent, in the liver[593]. ApoA-IV is found mainly associated with chylomicrons, in lower amount in HDL, and as a free plasma protein. In HDL, ApoA-IV stimulates LCAT, thereby facilitating HDL remodeling. Free plasma ApoA-IV facilitates cholesterol efflux from cells and has anti-oxidant properties. Transgenic overexpression of apoA-IV reduces atherosclerosis in

mice, and plasma apoA-IV levels are inversely correlated with atherosclerosis in humans. The ApoA-IV gene contains LXREs; however, LXR agonists stimulate its expression only in hepatocytes and not in the intestine. In vivo, T0901317 increases HDL-associated apoA-IV, presumably of hepatic origin since T0901317 has no effect on intestine-derived chylomicron apoA-IV[594].

LXRs regulate plasma lipoprotein remodeling enzymes LXR agonists stimulate the expression of CETP and PLTP, and both enzymes play important roles in lipoprotein remodeling and metabolism. CETP is synthesized in the liver and circulates in plasma as a HDL-associated protein. CETP exchanges cholesterol esters from HDL to apoB-containing lipoproteins in exchange for triglycerides. As discussed in the previous section, CETP remodeling may facilitate cholesterol uptake by the liver. LXR agonists increase CETP mRNA levels in the liver as well as plasma protein concentration[595]. The lipid transfer activity of CETP and the fact that it is under LXR regulation is a likely explanation for why LXR agonists dramatically increase plasma HDL-C in animals lacking CETP (e.g. mice) but have little to no effect on HDL in CETP expressing species (e.g. humans)[596]. In fact, LXR agonists have actually been shown to raise LDL-C levels in two CETP positive Syrian hamsters and cynomolgus monkeys[597] as well as in transgenic mice expressing human CETP[598].

PLTP mediates the transport of phospholipids from apoB-containing lipoproteins to HDL or lipid-poor apoA-1 as well as the transfer of phospholipids between HDL subfractions. The net effect of PLTP on HDL is the generation of lipid-poor small HDL; whether this is beneficial for cholesterol transport and atherosclerosis is debatable[599]. Nevertheless, T0901317 treatment increases PLTP mRNA in liver, adipose and intestine

resulting in greater plasma PLTP activity which is accompanied by increased HDL phospholipid content[600]. PLTP also contributes to VLDL assembly in hepatocytes[601], therefore, the stimulation of PLTP by LXR may contribute to LXR agonist dependent increases in VLDL secretion.

LXRs and Inflammation

In addition to their key role in lipid metabolism, LXRs also influence the inflammatory response. Indeed, the engulfment of pathogens or phagocytized cells increases intracellular cholesterol levels, and given the precedence of LXRs for maintaining cholesterol balance, it's not surprising that LXRs limit inflammation. Furthermore, there's increasing evidence that infections block LXR signaling pathways. Work by Castrillo et al[602]. demonstrated that activation of toll-like receptor (TLR)-3 and -4 by microbial ligands block the induction of LXR target genes including ABCA1 in cultured macrophages and in aortic tissue in vivo, thereby repressing cholesterol efflux. Crosstalk between LXR and TLR signaling is mediated by IRF3, a specific effector of TLR3/4 that inhibits the transcriptional activity of LXR. The association of bacterial infections and atherosclerosis, while controversial have long been recognized [140], The findings by Castrillo et al. reveal a mechanism whereby pathogens may modulate macrophage cholesterol metabolism and atherosclerosis. Indeed, *C. pneumoniae*-induced atherosclerosis, which can be reduced by TLR2,TLR4, or MyD88 deficiency, is accelerated in *apoE*$^{-/-}$ */Lxrα*$^{-/-}$ mice[143] indicating that LXRs play an important role in limiting the manipulations of macrophage cholesterol balance by infectious agents. LXR agonists were first shown to attenuate inflammation in macrophages stimulated with LPS or bacteria by blocking the increased expression of iNOS, COX2 and iL-6. These

results were also observed in the in the aortas of *apoE*$^{-/-}$ mice[603]. Since then, LXR activation has been found to reduce inflammatory gene expression and the inflammatory response in many cell types, including macrophages, lymphocytes, microglia, astrocytes and dendritic cells; as well as in mouse models of inflammatory diseases such as contact dermatitis, Alzheimer's, lupus, Mycobacteria *tuberculosis* infection, and autoimmune encephalomyelitis[604-609]. Several mechanisms have been proposed to account for the anti-inflammatory activity associated with LXR activation. The anti-inflammatory activity of LXRs may be through direct activation of anti-inflammatory target genes possessing LXREs (i.e. arginase II[610]) and/or indirect repression of other transcription factors that promote inflammation (i.e NFκB). The ability of LXR to limit the activity of other transcription factors is termed transrepression and this property is shared among several transcription factors, including PPARγ; where it has been perhaps more widely studied[544-546]. LXR transrepression was a model proposed a few years ago to account for the majority of the anti-inflammatory activity of LXR agonists. However, the mechanism(s) by which LXRs exert their anti-inflammatory effect is controversial. Recent data published by the same lab, as well as experiments preformed in our lab contradict the transrepression mechanism for LXR agonist-dependent transrepression. Both models are presented below.

Transrepression by LXRs has been most widely studied in mouse macrophages[1, 611, 612] where LXR agonists have been shown to block inflammatory pathways activated by lipopolysaccharide (LPS), interleukin (IL)-1b, tumor necrosis factor (TNF)α or interferon (INF)-γ. Ligand-dependent conjugation of SUMO2/3 to LXR, which is dependent on histone deacetylase 4 (HDAC4) SUMO E3 ubiquitin ligase activity, targets

the receptor to the promoters of a subset of LPS-inducible genes (Figure 1.3). Docking of SUMOylated LXR inhibits the release of the nuclear receptor corepressor (NCoR) complex upon LPS stimulation (i.e. derepression), thereby maintaining gene repression. Venteclef et al[613] have further investigated the mechanisms underlying the anti-inflammatory properties of LXRs in the hepatic acute phase response (APR). LXR agonists were found to trigger SUMOylation-dependent recruitment of the nuclear receptor to hepatic APR promoters, including C-reactive protein, and prevent the clearance of the NCoR corepressor complex upon cytokine stimulation (IL-1β and IL-6).

The use of fetal liver-derived macrophages from genetic knockout mice showed the NCoR and another corepressor complex, silencing mediator of retinoic acid and thyroid hormone receptor (SMRT), are required for nearly all the transrepression function of LXRs[612]. Moreover, the TLR4-induced turnover of NCoR requires the coronin 2A (CORO2A) component of the corepressor complex, which interacts with oligomeric nuclear actin[611]. SUMOylated LXRs bind to a conserved SUMO2/3-interaction motif in CORO2A and prevents actin recruitment[611]. Pro-inflammatory stimuli that induce CAMKIIγ-mediated phosphorylation of LXRs inactivate this transrepression pathway by promoting the de-SUMOylation of LXRs, thereby releasing LXRs from CORO2A[611]. Noting the requirement for NCoR in mediating the trans-repressing activity of LXRs, presumably the deletion of NCoR from the macrophage should increase inflammation. Instead, Glass and colleagues[614] found that there was a paradoxical anti-inflammatory phenotype in the macrophage-specific NCoR deficient mouse. This effect was attributed to the derepession of LXRs that results in the absence of NCoR. The release of basal LXR repressing activity increases the expression of genes that direct the biosynthesis of

palmitoleic acid and ω3-fatty acids. Increased ω3 fatty acid levels primarily inhibit NFκB dependent inflammatory responses by uncoupling NFκB binding and enhancer/promoter histone acetylation (Figure 1.5). While it is well known that LXR agonists have strong anti-inflammatory effects both *in vitro* and *in vivo* the mechanism(s) responsible for these effects are still uncertain. Whether LXRs exert anti-inflammatory effects via SUMO-dependent transrepression, through the increased biosynthesis of fatty acids, or by another yet to be identified mechanism remains to be determined.

Athero-protective effects of LXR agonists	**References**
Blocks the development of atherosclerosis in animal models of cardiovascular disease	529, 548-555, 615-617
Promote regression of existing atherosclerotic plaques	555, 556
Promote reverse cholesterol transport *in vivo*	561
Reduce inflammation *in vivo*	603, 604, 606, 608, 618, 619
Inhibit SMC proliferation *in vitro* and *in vivo*	620, 621
Stimulate cholesterol efflux in macrophages and SMC	621, 622
Inhibit platelet aggregation in vitro and reduce thrombi stability *in vivo*	552
Promote LDL receptor degradation through induction of IDOL	568, 623
Block expression of cytokines induced by pro-inflammatory mediators in macrophages, endothelial cells, and SMCs	603, 620, 621, 624
Inhibit LPS-stimulated surface expression of ICAM-1, VCAM-1, and E-Selectin on endothelial cell	1, 604, 625
Inhibit LPS-induced expression of iNOS, COX-2, osteopontin and MCP-1 in macrophages	1, 603, 625

Table 1.1 Atheroprotective activities of LXR agonists.

treatment	Animal model	Effect on lesion area	Effect on lesion composition	Effect on plasma lipids
T0901317[549, 552, 626]	*Ldlr*$^{-/-}$ WD	↓ lesion; ↑ regression	↓macrophages. ↑ABCA1 mRNA, ↑collagen	↑ TG, VLDL-C ↑ HDL-C
T0901317[627]	*apoE*$^{-/-}$ HFHC	↓ lesion ↑ regression	↑ABCA1, NPC1 mRNA	↑ TG, VLDL-C ↑ HDL-C
T0901317[555]	apoE*3Leiden, WD	↓ lesion	↓ E-selectin, ICAM-1, CD44	
T0901317[555]	apoE*3Leiden, RD	↑ regression	↑ABCA1, caspase-3, BAX, CCR7, ABCG1	
T0901317[628]	Lxrα$^{-/-}$ *Ldlr*$^{-/-}$, WD	No effect		
T0901317[628]	Lxrβ$^{-/-}$ *Ldlr*$^{-/-}$, WD	↓ lesion		
GW3965[548]	*Ldlr*$^{-/-}$ WD	↓ lesion		↓ TC
GW3965[548]	*apoE*$^{-/-}$ chow	↓ lesion	↑ ABCA1	↑ TG, ↓ VLDL-C
GW3965[558]	Lxrα$^{-/-}$ /*apoE*$^{-/-}$, WD	↓ lesion		No change in plasma TGs
	Lxrα$^{-/-}$ *Ldlr*$^{-/-}$, WD[628]	↑ lesion		
	Lxrβ$^{-/-}$ *Ldlr*$^{-/-}$, WD[628]	No effect		
Lxrα$^{-/-}$ *Ldlr*$^{-/-}$ BMT[628]	*Ldlr*$^{-/-}$ WD	↑ lesion		
Lxrα$^{+/+}$ *Ldlr*$^{-/-}$ BMT[628]	Lxrα$^{-/-}$ *Ldlr*$^{-/-}$ WD	↓ lesion		
Lxrα$^{-/-}$ β$^{-/-}$ BMT[569]	*Ldlr*$^{-/-}$ or *apoE*$^{-/-}$	↑ lesion	↑ cholesterol accumulation in macrophages	No change
Lxrα$^{-/-}$ β$^{-/-}$ BMT; T0901317[549]	*Ldlr*$^{-/-}$ WD	↑ lesion (vehicle) No effect of T0901317		
Macrophage LXRα Tg[560]	*Ldlr*$^{-/-}$, SSD	↓ lesion	↑ efflux and ↓iNOS production in macrophages	No change

Table 1.2 LXR activity and atherosclerosis in mouse models BMT, bone marrow transfer; WD, western diet; RD, regressive cholesterol-depleted diet; SSD, semi-synthetic diet, 0.02% cholesterol; TC, total cholesterol; TG, triglyceride; Tg, transgenic; HFHC, high fat, high cholesterol

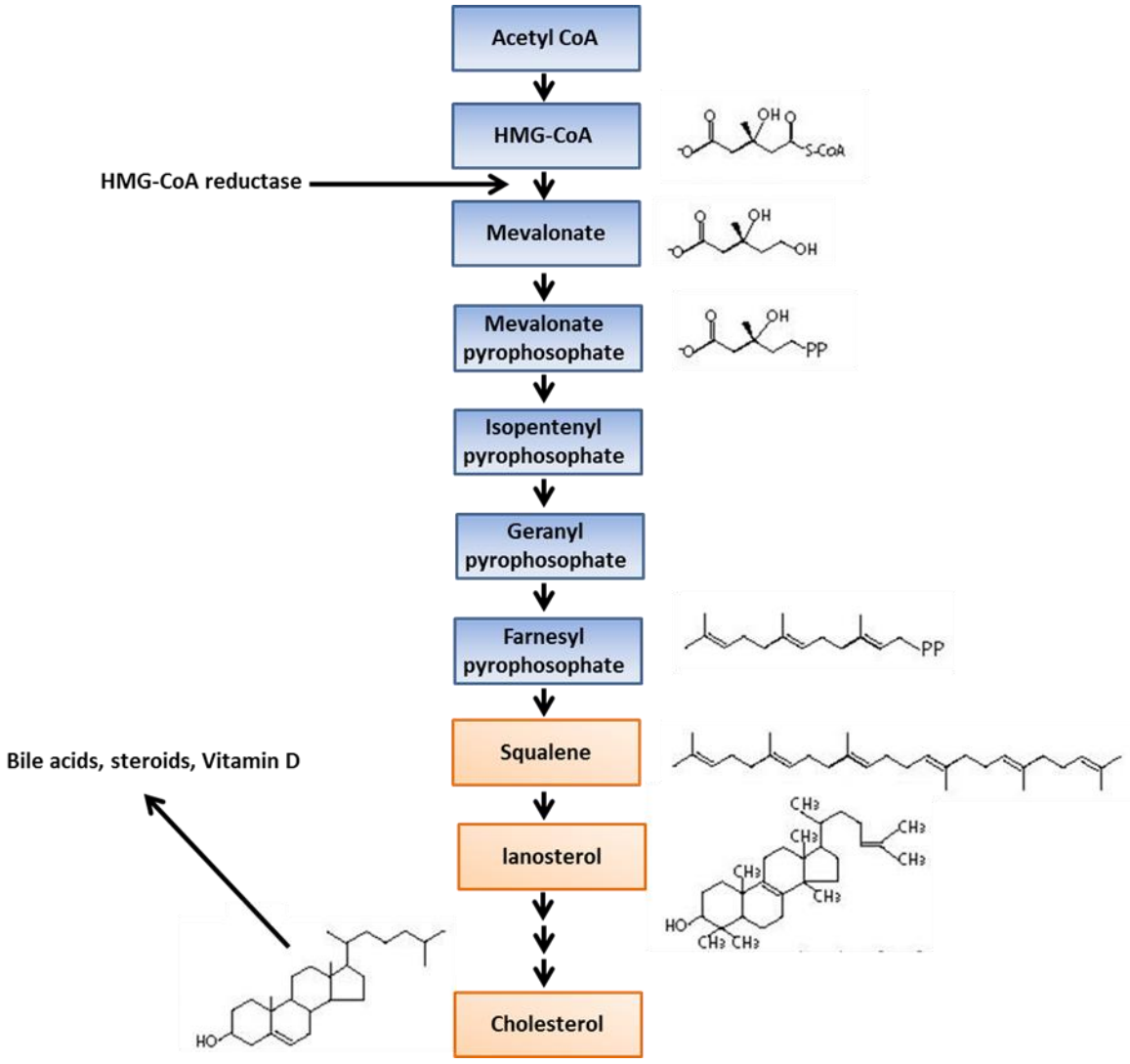


Figure 1.1 cholesterol biosynthesis pathway. See text for details

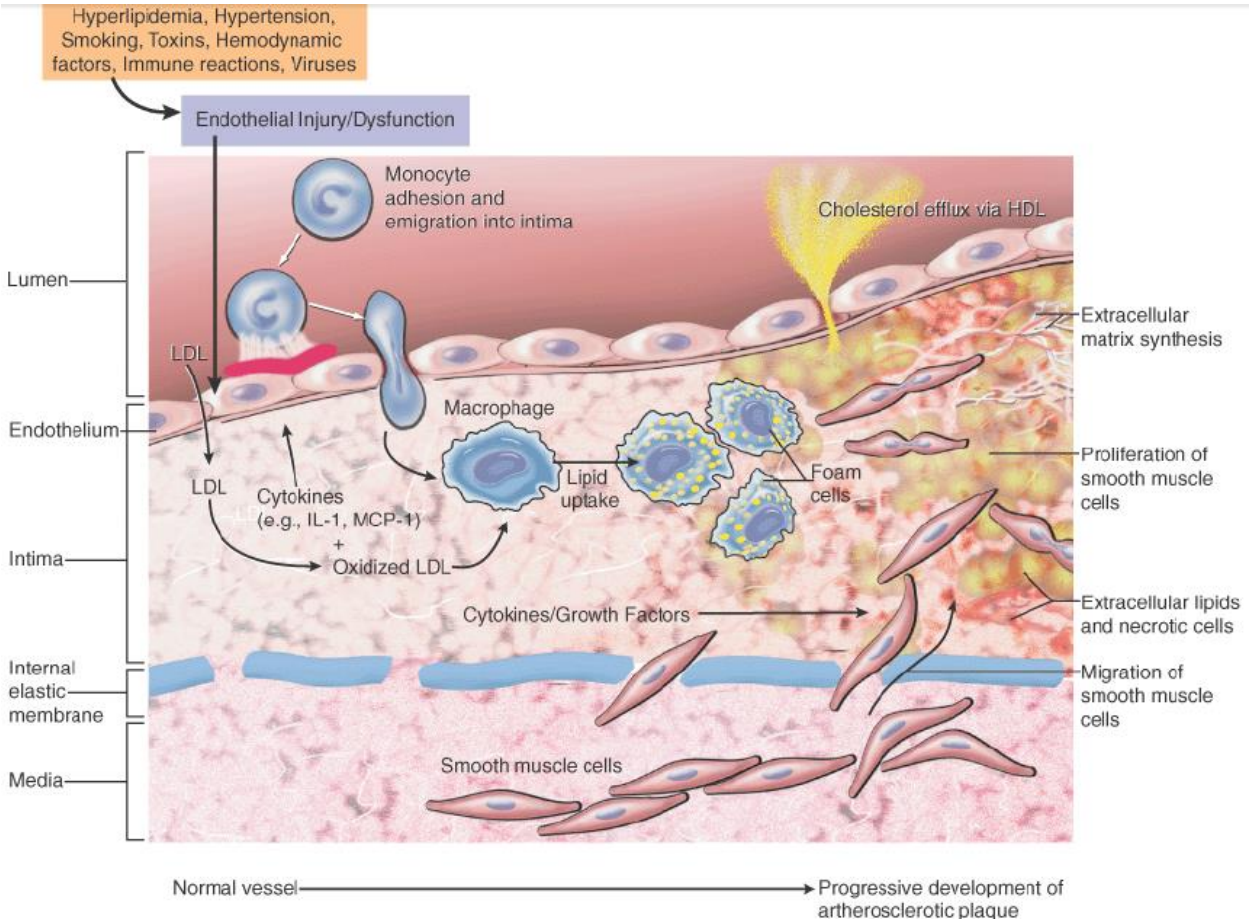
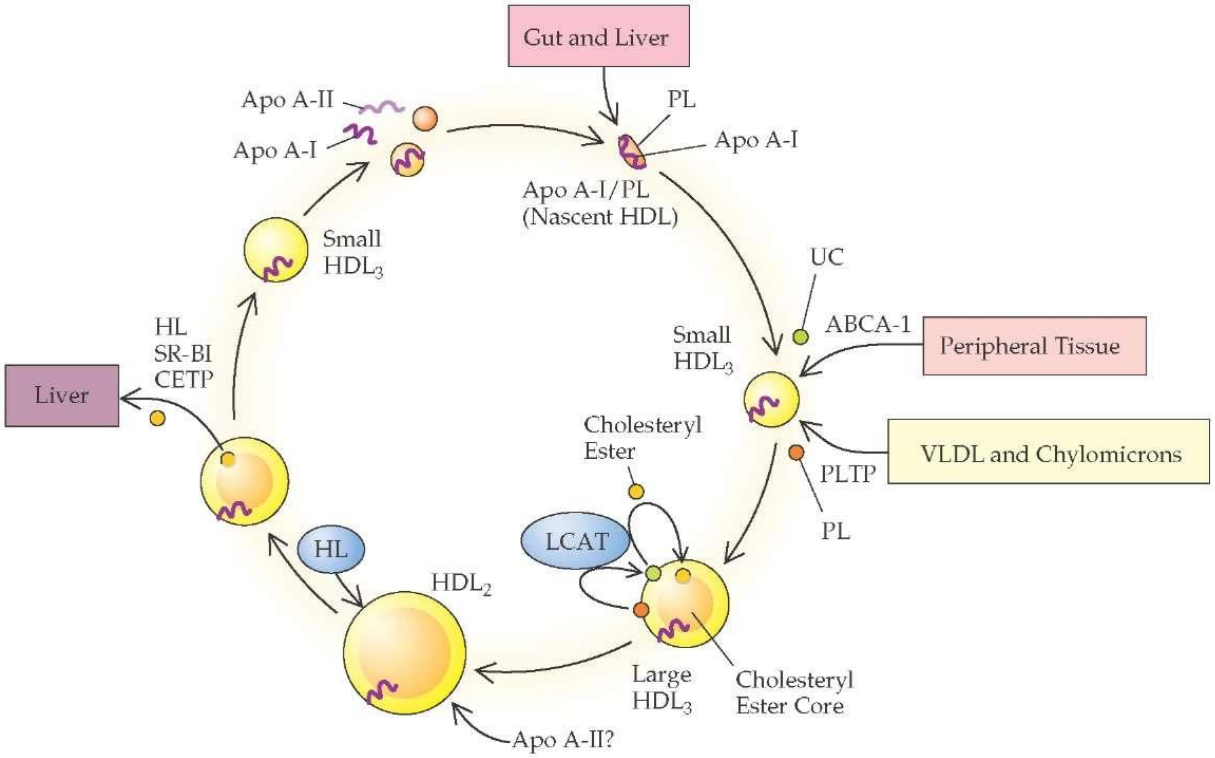


Figure 1.2 ***Progression of Atherosclerosis***

Figure 1.2 ***Progression of Atherosclerosis*** Chronic endothelial cell injury leads to increased permeability of the endothelium and leukocyte adhesion and entry. Oxidation of lipoproteins accumulated in the arterial intima also promotes leukocyte adhesion and entry. Migration of monocytes into the arterial intima is followed by differentiation into macrophages which then engulf ox-LDL and ultimately progress into foam cells as the engulfment of lipids becomes excessive. Necrosis of foam cells results in release of extracellular lipids. Factors released from platelets, macrophages and endothelial cells in the forming lesion induce SMC recruitment from the media. Migrating SMCs proliferate and there is an increased production of ECM and collagen in an attempt to stabilize the atherosclerotic lesion. The plaque can then calcify, occlude, break off or hemorrhage. LDL, low-density lipoprotein; SMC, smooth muscle cell; ECM, extracellular matrix; IL-1, interleukin-1; MCP-1, monocyte chemoattractant protein-1; HDL, high-density lipoprotein



Adapted from treatments for dyslipidemia http://what-when-how.com

Figure 1.3 ***HDL formation and degradation***

Figure 1.3 ***HDL formation and degradation.*** HDL begins as an ApoA1 phospholipid complex secreted by the liver and intestine. Unesterified cholesterol and phospholipids are added to nascent HDL via ABCA1 and PLTP to begin the formation of the smaller HDL3 particle. LCAT transfers a free fatty acid from HDL-associated phospholipids to unesterified cholesterol forming cholesteryl esters, which migrate to the HDL core. This process results in larger, more buoyant HDL3 particles and then progress to even larger HDL2 particles. CETP transfers cholesteryl esters from HDL to ApoB-containing lipoproteins in exchange for triglycerides, resulting in smaller HDL particles. Hepatic lipase hydrolyzes the phospholipid and triglyceride in the HDL2 particle, promoting the decrease in size and density. Recycling of apoA1 causes the process to repeat itself. The role of ApoAII in this process in humans is not clear. ABCA1-ATP-binding cassette transporter A1; CETP – cholesteryl ester transfer protein; LCAT – lecithin-cholesterol acyltransferase; LPL – lipoprotein lipase; PL – phospholipid; PLTP – phospholipid transfer protein; SR-B1 – scavenger receptor B1; UC-unesterified cholesterol

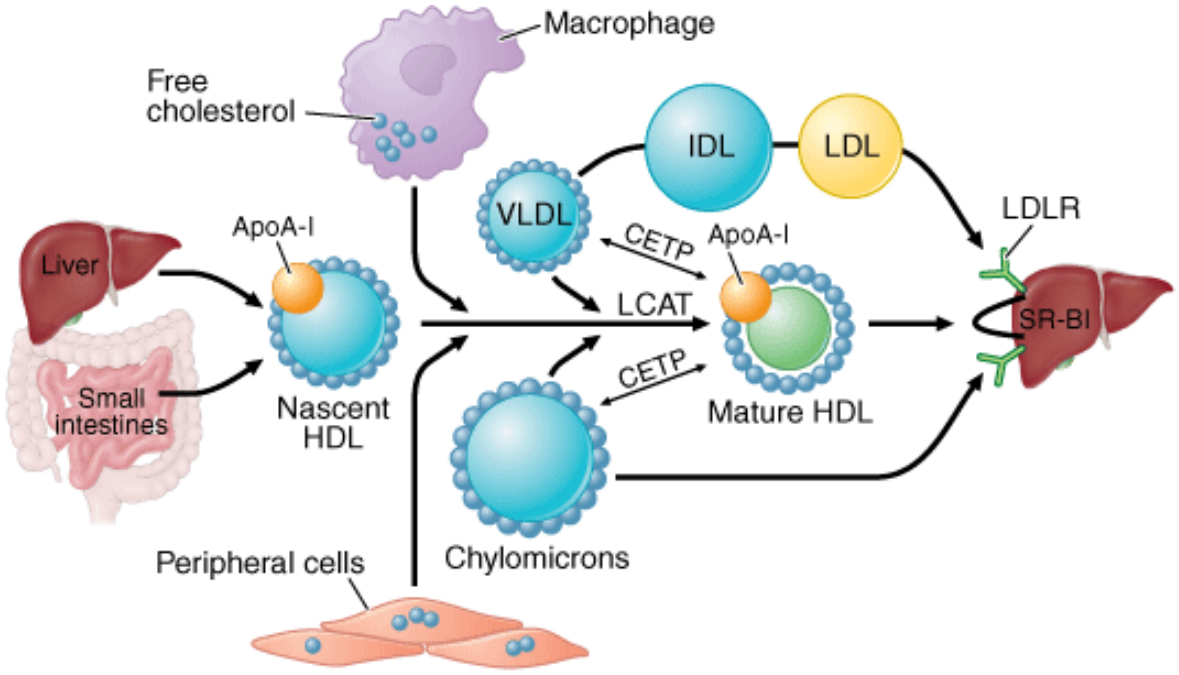


Figure 1.4 ***HDL metabolism and reverse cholesterol transport***

Figure 1.4 HDL metabolism and reverse cholesterol transport. The liver and intestine produce nascent HDLs. Free cholesterol is acquired from macrophages and other peripheral cells and esterified by LCAT, forming mature HDLs. HDL cholesterol can be selectively taken up by the liver by SR-B1. Alternatively, HDL cholesteryl ester can be transferred to apoB-containing lipoprotein particles (VLDL, LDL and chylomicrons) in exchange for triglycerides. ApoB-containing lipoproteins can be taken up by the liver via LDLR and other lipoprotein receptors. LCAT, lecithin-cholesterol acyltransferase; CETP, cholesteryl ester transfer protein; VLDL, very-low density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; LDLR, low-density lipoprotein receptor; TG, triglyceride

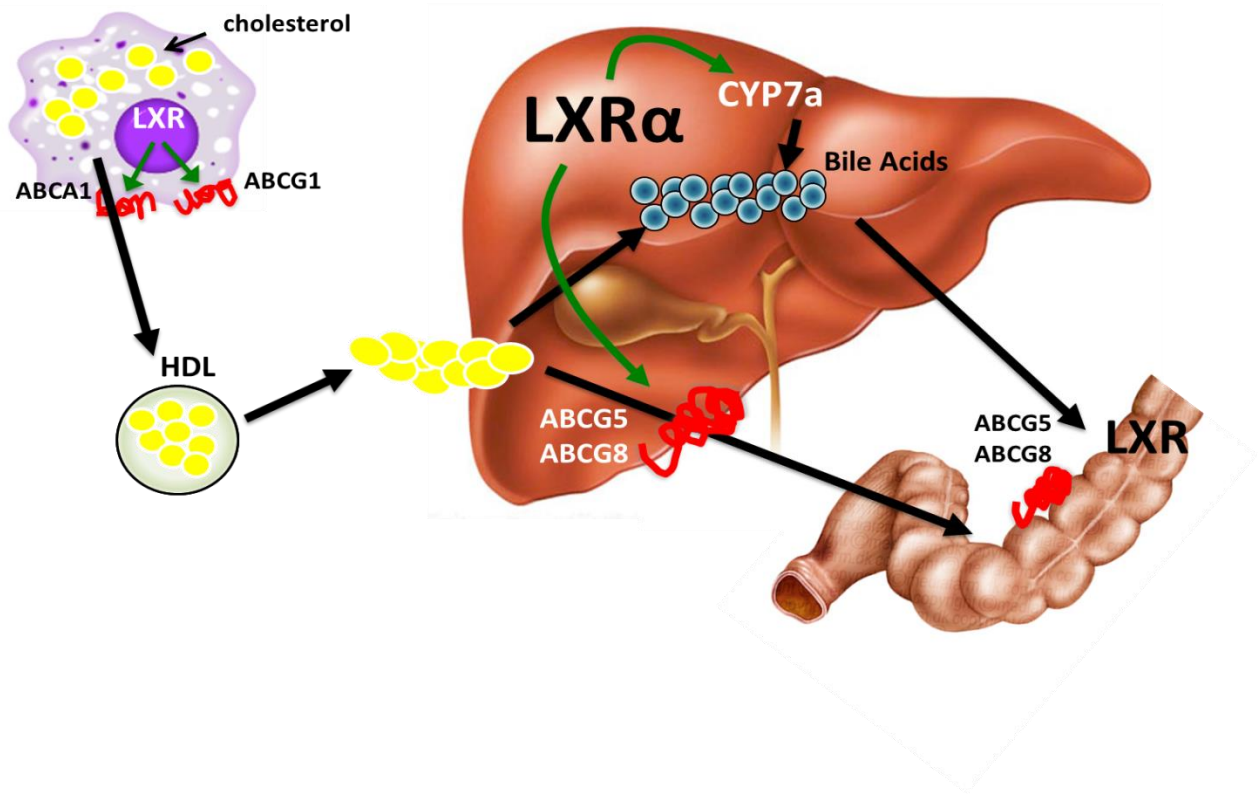
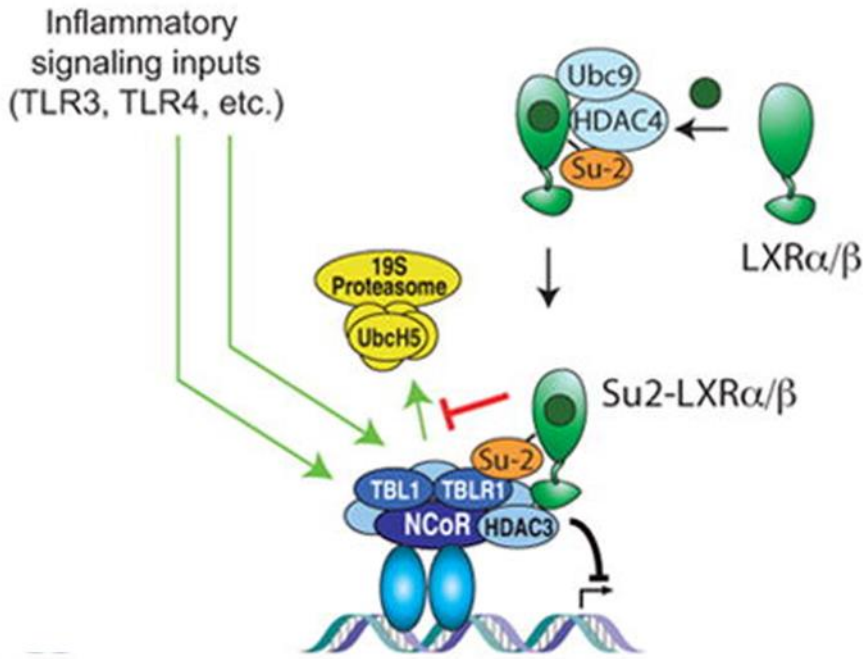


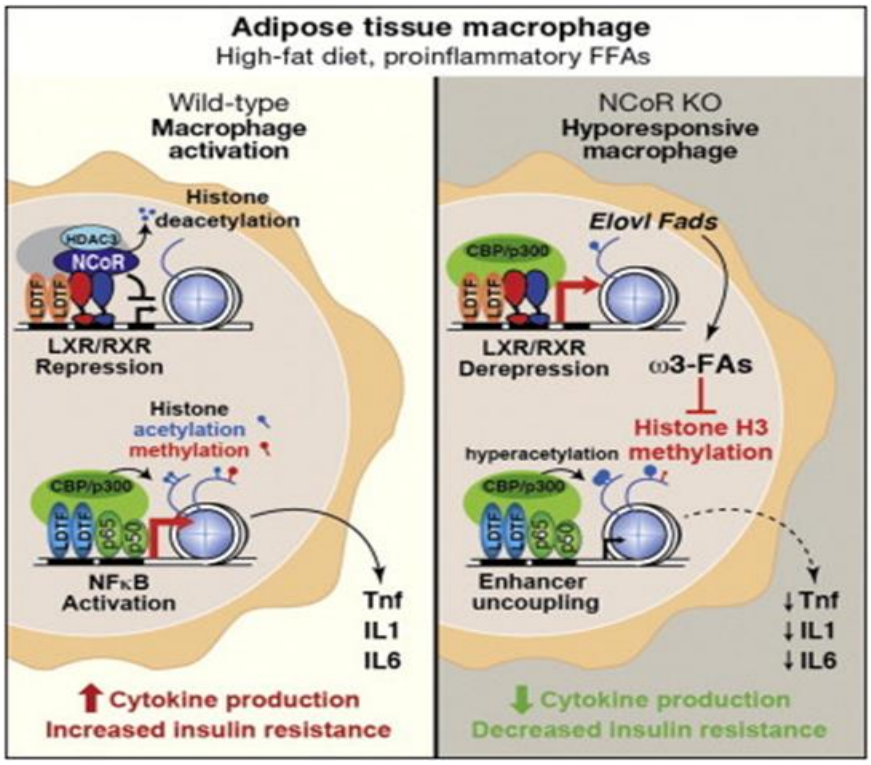
Figure 1.5 ***LXR regulated reverse cholesterol transport.*** See text for details.

HDAC4: SUMO2/3 pathway



iNOS,
IL-1b

Figure 1.3 ***SUMO-dependent transrepression by LXR agonists.*** LXR agonist binding induces a conformational change in LXR that alters the specific configurations of the lysine residues that serve as SUMO acceptor sites. Upon conjugation with SUMO2/3 by HDAC4, LXRs dock with the NCoR complex positioned on LPS-responsive genes. Signal-dependent (LPS, TNFα, IL-1b, etc) clearance of NCoR from target promoters involves the activation of the ubiquitin E3 ligase activities of the Tbl1 and TblR1 proteins that are core components of the NcoR complex, leading to the recruitment of the Ubc5/19S proteasome and the subsequent ubiquitylation and dismissal of the NCoR complex. SUMOylated LXR prevents NCoR dismissal following a pro-inflammatory stimulus.



Graphical abstract from Li et al.[614]

Figure 1.5 ***NCoR Repression of LXRs Restricts Macrophage Biosynthesis of Insulin-Sensitizing Omega 3 Fatty Acids***

Chapter 2: Macrophage Independent Regulation of Reverse Cholesterol Transport by Liver X Receptors

The work presented in this chapter is my manuscript that was originally submitted for review to the Journal of Arteriosclerosis, Thrombosis and Vascular Biology in February, 2014. We have addressed the reviewers' comments and a revised version, which is presented herein, was re-submitted for review in April 2014. This work was greatly assisted by Jerry Angdisen, who provided valuable help with the in vivo RCT experiments. Additional data that was not submitted with the revised manuscript has been included in this version. In addition, discussion of certain results that were not included in the re-submitted document is emphasized in italics.

2.1 ABSTRACT AND SIGNIFICANCE

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 (LXRα and LXRβ) 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 (macrophage, liver, and intestine) 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 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*.

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.

SIGNIFICANCE

The liver X receptors, LXRα and LXRβ, 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.

2.2 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 development[629]. Excess plasma cholesterol accumulates in macrophages lodged in blood vessel walls which along with an associated inflammatory response initiate the formation of atherosclerotic lesions[257]. Statin therapy is highly effective for lowering disease-causing low-density lipoprotein (LDL) cholesterol thereby reducing morbidity and mortality associated with CVD[630]. Nevertheless, the residual risk for major cardiac events remains high for patients receiving LDL lowering therapies prompting the search for complementary therapeutic approaches[631]. 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 HDL[632]. Recent clinical trials with cholesteryl ester transfer protein (CETP) inhibitors and niacin, however, have failed to demonstrate clinical benefits of increasing HDL cholesterol[428, 633]. 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[366, 412]. The ability of HDL to promote cholesterol efflux from macrophage foam cells within atherosclerotic lesions was one of its earliest recognized functions[373, 634]. Importantly, cholesterol efflux from foam cells has been shown to increase macrophage egression and to reduce lesion burden in animal models of cardiovascular disease[380, 635, 636]. Measuring the dynamic rate of macrophage cholesterol efflux, therefore, may be a better predictor of the anti-atherogenic effects of novel HDL-targeted therapies[371].

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[375, 637]. Recent studies have also described hepatic-independent pathways for cholesterol excretion[391]. Studies in animal models indicate that measurements of RCT can strongly predict the effect of genetic and pharmacological manipulations on atherosclerosis[638]. 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 status[371]. 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[381, 639].

Integral to the regulation of RCT are the liver X receptors, LXRα (NR1H3) and LXRβ (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[524, 583, 640]. Treatment of animals with LXR agonists results in changes in gene expression promoting the efflux of cholesterol from peripheral cells such as macrophages, the excretion of cholesterol from the liver, and the inhibition of cholesterol absorption in the intestine[640]. 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[517, 641]. 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)[640]. 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[642, 643]. 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[638, 644, 645]. LXR also regulates expression of ABCG5 and ABCG8, two half-transporters that dimerize to form an additional cholesterol transporter[575, 646]. 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)[34]. Genetic deletion of ABCG5/G8 or deletion of LXRα in the liver largely blocks the ability of LXR agonists to stimulate fecal excretion of cholesterol[529, 573]. 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[529, 548, 549, 628].

Treatment with LXR agonists also increases plasma HDL cholesterol[529, 647] 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 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[529, 579] . Not unexpected then is the observation that

an intestinal-specific LXR agonist increases RCT[648]. 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[549, 560, 569]. 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.

2.3 RESULTS

Macrophage LXR is not necessary for LXR agonist-dependent RCT.

LXR activity in the liver and the macrophage is thought to contribute to RCT[649] 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 ^{3}H-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.[561]. For these studies we used C57BL/6J (LXR^{+}) and *Lxrα*$^{-/-}$/*Lxrβ*$^{-/-}$ (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 ^{3}H sterol was quantitated in the plasma at 6, 24 and 48 hours after injection. At completion of the experiment (48 hours) the amount of ^{3}H-sterol in the feces and liver was determined. In preliminary experiments we determined 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 DKO mice and at least 10 times above the reported EC_{50} (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 2.1A-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 2.1A-B). Similarly, the ability of T0901317 to increase macrophage cholesterol efflux in Mac^{LXR+} /DKO mice is decreased by 70% (Figure 2.1A) and agonist stimulated fecal excretion is completely blocked in these animals(Figure 2.1B). 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 2.1C). 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 ^{3}H-cholesterol in the plasma or the feces (Figure 2.1A-B). Little or no differences among the groups are seen when hepatic levels of ^{3}H-sterols were examined (Figure 2.2). 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 ^{3}H-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 2.1D, pretreatment of mice with T0901317 significantly increases ^{3}H-cholesterol in the plasma by 60 minutes. Even at these short time points, however, the LXR genotype 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 ^{3}H-cholesterol in the plasma *in vivo* is

consistent with studies *in vitro* demonstrating that ABCA1 expression and cholesterol efflux is actually slightly increased in DKO macrophages (Figure 2.3A and B). In the absence of agonists LXRs repress transcription by interacting with corepressors and this activity is lost upon genetic deletion[650]. 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 2.1C).

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 intestine[579]. Consistent with an LXR agonist-dependent increase in HDL cholesterol (Table 2.1), plasma from T0901317 treated C57BL/6J (LXR+) mice has increased cholesterol acceptor activity *in vitro* when ^{3}H-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 2.4A). 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 2.4B). For experiments with FPLC purified HDL, peak HDL fractions were pooled (Figure 2.5) and normalized by the amount of apolipoprotein AI (APOAI) as determined by Western blot analysis (Figure 2.6). Western blot analysis of FPLC purified HDL was consistent with APOA1 levels quantified in pooled plasma samples as well (data not shown). Using APOA1 as a relative measure for particle number, HDL from agonist treated C57BL/6J accept greater amounts of macrophage cholesterol compared to DKO mice (Figure 2.4B). 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 ^{3}H-cholsterol 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 (Mac^{LXR+} /LXR+) and multiple tissues were harvested at 48 hours post injection to determine if agonist treatment produces a net loss in tissue-associated ^{3}H-sterols. As shown in Figure 2.4C, a significant agonist-dependent decrease is observed in white adipose suggesting that fat tissue may make an important contribution to LXR-stimulated accumulation of cholesterol in the plasma and feces. T0901317-dependent changes in ^{3}H-sterol levels were not observed in other tissues (Figure 2.4C). Importantly, the decrease in adipose ^{3}H-sterol levels could result from increased LXR transcriptional activity in fat cells, the improved acceptor activity of HDL or both. *An additional question that has arisen from this particular in vivo RCT assay is how much 3H-labeled cholesterol leaves the injected macrophages and how is the radiolabeled cholesterol distributed throughout the body. Our studies indicate that in fact at 48 hours the majority of the ^{3}H cholesterol leaves the injected macrophages and is redistributed among various cholesterol stores in the body. The amount of radioactivity remaining in re-extracted macrophages was ~4% of CPM injected (data not shown) and extrapolating by the % tissue mass/body mass approximately 30% of the radiolabeled 3H was recovered in the tissues that were*

sampled (muscle (8%,), liver (6%), adipose (~6%), testes (~1%) and feces (7%)) (Figure 2.4C).

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 LXRα impairs the accumulation of macrophage-derived cholesterol in both the plasma and in the feces[529]. To further investigate the contribution of liver LXR activity to RCT, liver-specific knockout LXRα (LivKO) mice[529] and floxed littermate controls (carrying the floxed allele without albumin CRE) were placed on a standard chow diet with or without 0.2% cholesterol. LXRα is the major LXR subtype expressed in the liver[541] and the ability of T0901317 to increase plasma triglycerides and to induce expression of hepatic ABCG5, ABCG8 and ABCA1 is significantly impaired in LivKO mice[529] (Table 2.1 and Figure 2.7). 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 2.1). 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[529, 541] (Figure 2.8A). Hepatic triglycerides, however, are not increased (Figure 2.8B). The increase in hepatic cholesterol measured in LivKO mice, however, does not result in a significant increase in liver damage (Figure 2.8 C-D and Figure 2.9), markers of inflammation or markers of endoplasmic reticulum stress (Figures 2.10 and 2.11). 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 ^{3}H-cholesterol in the plasma of T0901317 treated

LivKO and littermate controls is significantly increased at 24 and 48 hours (Figure 2.12A) indicating that liver LXRα activity is not required for agonists to increase accumulation of ^{3}H-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 2.11B) a result consistent with decreased agonist-dependent regulation of ABCG5 and ABCG8 in the livers of these animals (Figure 2.7). 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 2.12C-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 2.13A and Table 2.1) despite similar increases in the intestinal mRNA levels of ABCA1 (Figure 2.14). 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 2.13B and Figure 2.5). 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 ability of LXR agonists to promote macrophage-derived cholesterol accumulation *in vivo* is largely derived from systemic effects on HDL and independent of macrophage LXR activity.

Although agonist-dependent fecal excretion is blocked in standard chow diet fed LivKO mice, surprisingly a corresponding accumulation of ^{3}H-cholesterol in the livers of agonist treated LivKO animals is not detected under these conditions (data not shown). The reason for this finding is not apparent. One possibility is that the livers of T0901317 treated LivKO animals take up less cholesterol compared to floxed controls. Gene expression analysis, however, did not reveal any significant differences in the expression of lipoprotein receptors in the livers of T0901317 treated LivKO and floxed animals (Figure 2.15). We note that others have shown that changes in biliary cholesterol secretion do not always directly reflect changes in hepatic cholesterol levels. For instance, adenoviral over expression of ABCG5 and ABCG8 in the liver increases fecal sterol excretion without changing hepatic cholesterol[651]*. Future studies should be considered to investigate the effect of Liver LXRα deletion on hepatic uptake of HDL-cholesterol and HDL turnover. Such studies could provide insight into the paradoxical observation that LXR agonists increase plasma ^{3}H accumulation without raising liver ^{3}H levels LivKO mice.*

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[485, 652]. As shown in Figure 2.13C and 2.13D, 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 2.13C). The increase in HDL-phospholipid levels is consistent with studies demonstrating that LXR agonist treatment increased HDL particle size[529, 653].The effect of agonist treatment on HDL-phospholipid levels, however, is lost in 0.2% cholesterol diet challenged LivKO animals (Figure 2.13D).

Phospholipid transfer protein (PLTP) is a HDL-bound protein that plays a major role in regulating HDL size and phospholipid composition through its phospholipid transfer activity[654]. PLTP mRNA levels have been shown to be regulated by LXR[600] however we did not detect significant differences in plasma PLTP activity between floxed and LivKO mice on either dietary condition (Table 2.2). *We did, however, detect a loss of LXR agonist stimulated PLTP mRNA expression in the intestine in 0.2% cholesterol diet fed LivKO mice. Two forms of PLTP have been shown to exist in plasma[655, 656]. The "active" form has the ability to transfer phosphatidylcholine from phospholipid vesicles to HDL and is detected by the activity assay used in this study. The second inactive form constitutes approximately 70% of the PLTP protein mass in human plasma[656]. The two PLTP pools are associated with different types of lipoprotein particles, suggesting that PLTP activity in circulation is modulated by the composition of lipoproteins. While we did not observe a difference in plasma PLTP activity among the groups, whether this loss of agonist stimulated PLTP expression in the intestine of cholesterol fed LivKO mice plays a role in HDL metabolism, perhaps by increasing the amount of the non-enzymatically active PLTP should be addressed in future studies.*

FPLC-purified HDL from chow and 0.2% cholesterol diet fed LivKO mice and floxed controls were analyzed for phospholipid composition by mass spectrometry. As

shown in figure 2.16, in the floxed controls on either chow or 0.2% cholesterol diet, LXR agonist treatments increases the amount of all phospholipid species present in HDL particles. In the chow fed LivKO mice, however, LXR agonist increases the appearance of short chain fatty acid containing phospholipid species and there is no agonist-stimulated increase in long chain, unsaturated fatty acid containing species. This is consistent with the role of LXR in regulating the expression of genes encoding for fatty acid elongases and desaturases[524]. Conversely, the ability of LXR agonists to increase the appearance of all phospholipid species is lost in 0.2% cholesterol diet fed mice.

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 macrophage cholesterol efflux, we took advantage of the observation that LXR agonist-dependent increases in HDL cholesterol are lost in CETP transgenic mice[657]. CETP facilitates the transfer of cholesterol esters from HDL to apolipoprotein B (APOB) containing particles and decreases HDL cholesterol levels[596]. 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[595, 598] (Figure 2.17A and B). Indeed, treatment of CETP transgenic mice with T0901317 decreases HDL cholesterol by approximately 25% and raises the amount of cholesterol associated with APOB containing lipoprotein particles (Figure 2.18A and B and Table 2.1). 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 ^{3}H-cholesterol loaded C57BL/6J (LXR^{+}) BMM as described in previous experiments. Consistent with a critical role for HDL in promoting macrophage cholesterol efflux, the

amount of ^{3}H-cholesterol in the plasma 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 2.18C). Similarly, unfractionated plasma and HDL particles purified by FPLC from T0901317 treated CETP transgenic mice do not exhibit increased efflux activity as is observed in with non-transgenic controls (Figure 2.18D-E). The ability of LXR agonists to increase HDL phospholipids, however, is not impaired in CETP transgenics (Figure 2.17C). 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 cholesterol efflux. In contrast to the inhibitory effect of CETP expression on the accumulation of macrophage-derived cholesterol is plasma, LXR agonist treatment increases fecal ^{3}H-sterol levels in both CETP transgenic and littermate controls (Figure 2.18F). Interestingly, CETP expression also results in a significant increase in fecal bile acids in vehicle treated cells (Figure 2.17D). Increased bile acid synthesis has previously been reported in CETP transgenic mice[658, 659]. Little or no difference was observed in hepatic ^{3}H-cholesterol levels among the groups (data not shown). Thus as observed with the LXRα liver-specific knockout mice (LivKO), it is possible to functionally sever the transfer of macrophage-derived cholesterol to HDL from subsequent fecal excretion.

2.4 DISCUSSION

The discovery that LXR agonists can promote macrophage cholesterol efflux *in vitro* via direct regulation of the genes encoding ABCA1, ABCG1 and APOE[570, 640] 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 agonists[549]. 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 2.19). 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 2.19). The LXR agonist used in these studies, T0901317, has been reported to modulate other nuclear receptors, at least *in vitro*[525, 526, 660]. 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 deficient in LXRs. Taken together these studies have important implications for understanding the potential therapeutic benefits of LXR agonists.

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[529, 579, 661]. 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 agonist[648] or intestinal-specific transgenic over expression of a hyperactive LXR (VP16-LXRα)[662] 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 2.19) most likely by increasing the production of immature nascent particles that have been shown to be preferred cholesterol acceptors[663-665]. This work 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[598, 657]. CETP, an enzyme that transfers cholesterol esters from HDL to APOB containing lipoprotein particles in exchange for triglycerides, is not expressed in rodents but the human gene used in this study is regulated by LXRs[595, 598, 666]. Importantly CETP activity in the plasma is increased following LXR agonist treatment, HDL levels are lower and plasma cholesterol accumulation measured during RCT assays *in* vivo 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 particles[667]. 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. Nevertheless, in CETP transgenic LXR agonist treatment still increases fecal excretion of macrophage-derived cholesterol mice. 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 APOB 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 ^{3}H-cholesterol in RCT assays without altering fecal sterol excretion[661]. Bi et al. suggest the small plasma HDL pool that remains in the liver ABCA1 knockout may be quantitatively sufficient to mediate the transport macrophage-derived cholesterol to the liver for excretion[661]. Our study with CETP transgenic mice together with the work of Bi et al. raise the possibility, at least under these experimental conditions, that the appearance of macrophage-derived in the plasma is a not a rate limiting step for fecal cholesterol excretion.

In contrast to CETP transgenic expression, liver-specific deletion of LXRα (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 2.6). Thus our analysis of CETP transgenic and LXRα 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 (LXRα and LXRβ) 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[668, 669]. 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 LXRα 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 2.18). 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[529, 548, 552, 628, 647]. Thus the ability of LXR agonists to regulate HDL metabolism can be influenced by dietary cholesterol levels. Interestingly, Kalaany et al. demonstrated that *Lxr*$\alpha^{-/-}$/*Lxr*$\beta^{-/-}$ are resistant to high fat diet-induced obesity, however, this resistance is only observed when the high fat diet also contains cholesterol[572]. 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[549, 560, 569]. 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 RCT[529]. Kappus et al. also reached a

similar conclusion in a recent study using mice with myeloid-specific double knockout of *Abca1* and *Abcg1*[670]. 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[603, 671]. 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 actiities in human macrophages[672] 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[529, 548, 673]. 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[553, 597].

Recent clinical trials with niacin[633] and CETP inhibitors[428] 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[371, 381, 412]. 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[483, 484]. 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 triglycerides[485]. 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.

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	*Lxrα*$^{-/-}$ *Lxrβ* $^{-/-}$	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†	58.0 ± 3.3	59.9 ± 3.4
		T0901317	216.9 ± 16.0*†	94.9 ± 12.0*†	197.2 ± 17.6*†
0.2% cholesterol	LivKO	Vehicle	166.7 ± 11.0†	50.3 ± 9.9	47.7 ± 7.2
		T0901317	167.9 ± 6.2	59.9 ± 3.8	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*

Table 2.1. Plasma Lipid Levels.*Statistically significant difference between vehicle and T0901317-treated animals of the same genotype (n = 6, $p \leq 0.05\%$). †Statistically significant difference between chow and 0.2% cholesterol diet fed mice of the same genotype and treatment (n = 6, $p \leq 0.05\%$). ** Statistically significant difference between C57bl6/J and *Lxrα*$^{-/-}$ *Lxrβ* $^{-/-}$ with the same treatment. Data are mean ± SEM.

Diet	**strain**	**Treatment**	**Intestine *PLTP* mRNA**	**Liver *PLTP* mRNA**	**PLTP activity (nmol transferred/min)**
Chow	Floxed	Vehicle	0.99 ± 0.09	0.49 ± 0.05	3.6 ± 0.1
		T0901317	1.48 ± 0.08*	1.6 ± 0.18*	4.3 ± 0.3*
Chow	LivKO	Vehicle	0.93 ± 0.06	0.43 ± 0.11	3.5 ± 0.2
		T0901317	1.57 ± 0.16*	0.37 ± 0.08	4.0 ± 0.2
0.2% cholesterol	Floxed	Vehicle	0.43 ± 0.06$^{\#}$	0.20 ± 0.02$^{\#}$	2.0 ± 0.3
		T0901317	0.97 ± 0.18*	0.55 ±0.11*$^{\#}$	3.3 ± 0.2*
0.2% cholesterol	LivKO	Vehicle	0.46 ± 0.05$^{\#}$	0.15 ± 0.01$^{\#}$	2.5 ± 0.3
		T0901317	0.44 ± 0.08$^{\#}$	0.15 ± 0.03	3.2 ± 0.3*

Table 2.2 LivKO PLTP mRNA and plasma activity. *Statistically significant difference between vehicle and T0901317-treated animals of the same genotype (n = 4-6, $p \leq 0.05\%$). $^{\#}$Statistically significant difference between chow and 0.2% cholesterol diet fed mice of the same genotype and treatment. (n = 4-6, $p \leq 0.05\%$).Data are mean ± SEM.

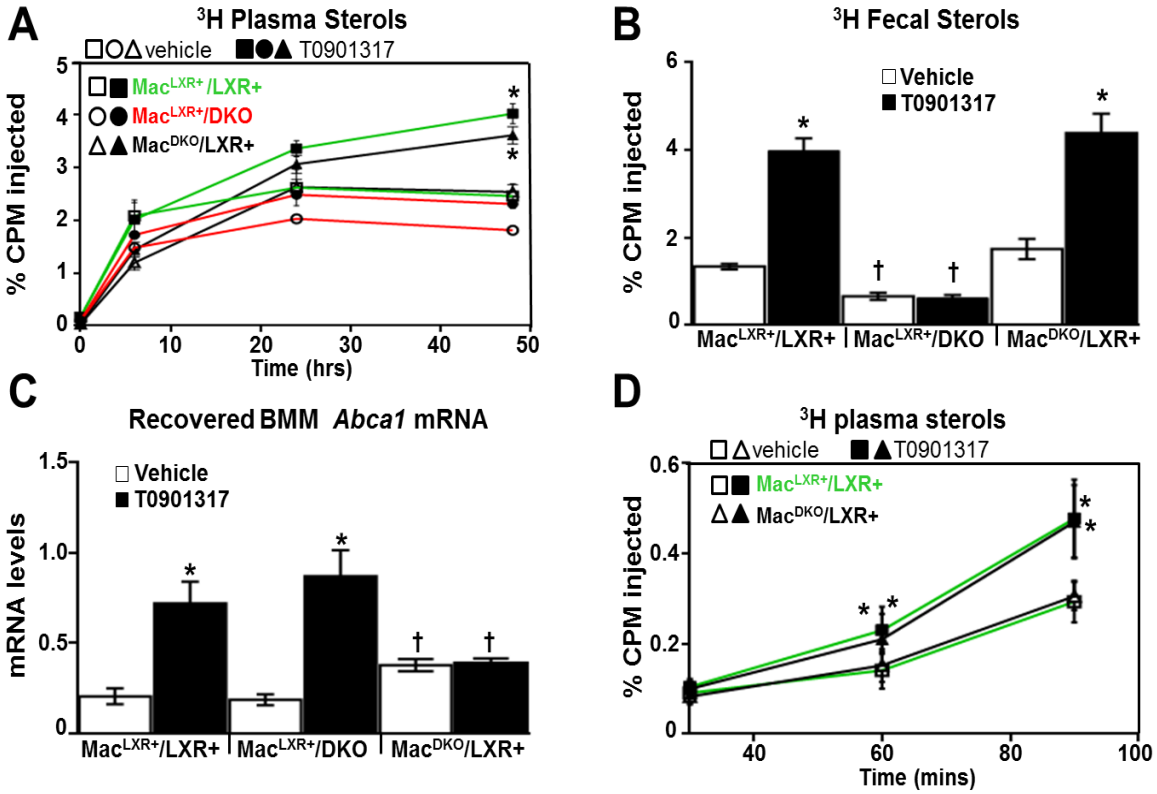


Figure 2.1 ***Macrophage LXRs are not required for RCT.***

Figure 2.1 ***Macrophage LXRs are not required for RCT***. ^{3}H-cholesterol and acetylated LDL-loaded C57BL6/J or LXRα$^{-/-}$/LXRβ$^{-/-}$ (DKO) BMMs were injected into C57BL6/J or DKO mice to generate Mac^{LXR+}/LXR+, Mac^{LXR+}/DKO, and Mac^{DKO}/LXR+ mice (see text). Animals were treated for 3 days with or without 10 mg/kg T0901317 (n=6/group), and the amount of ^{3}H 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 ± SEM. *Statistically significant difference between vehicle and T0901317-treated animals of the same genotype ($p \leq 0.05\%$). †Statistically significant difference between Mac^{LXR+}/LXR+, Mac^{LXR+}/DKO or between Mac^{LXR+}/LXR+ and Mac^{DKO}/LXR+ mice with the same treatment ($p \leq 0.05\%$).

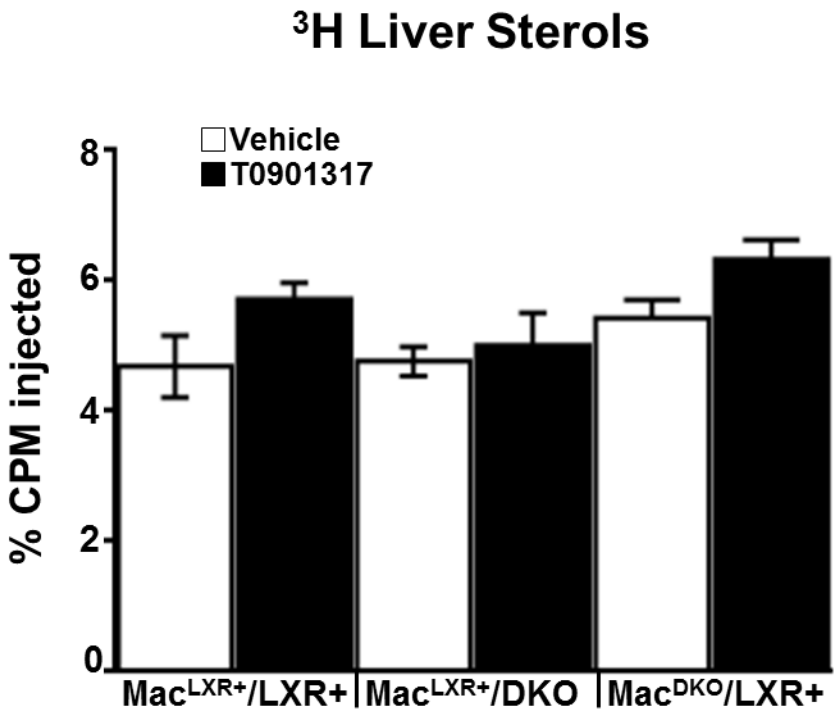


Figure 2.2 ***Liver ^{3}H sterol levels.***

Figure 2.2 ***Liver*** ^{3}***H sterol levels.*** The amount of ^{3}H-sterol in the livers of animals at the conclusion of the RCT experiment were determined as described in Material and Methods. Data are mean ± SEM.

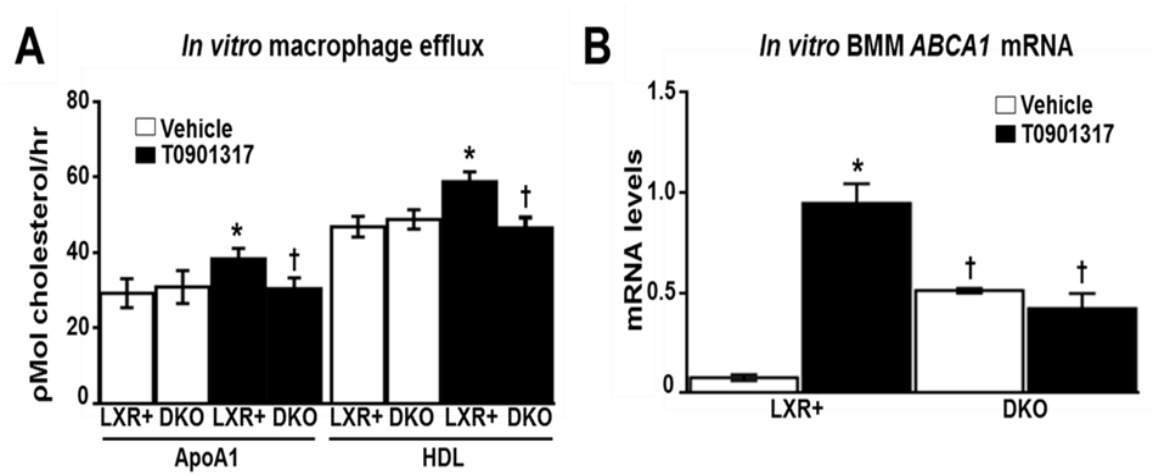


Figure 2.3 In vitro *cholesterol efflux in Lxrα$^{-/-}$ /Lxrβ$^{-/-}$ macrophages*.

Figure 2.3 In vitro *cholesterol efflux in Lxrα$^{-/-}$ /Lxrβ$^{-/-}$ macrophages.* A)Total RNA was isolated from LXR+ and DKO BMM treated with 1uM T0901317 in culture for 24 hours and mRNA levels of *Abca1* were measured by quantitative real-time PCR. **B)** *In vitro* macrophage cholesterol efflux was measured as described in Materials and Methods using ^{3}H-cholesterol labeled LXR+ or DKO incubated with 10ug/ml HDL or 10ug/ml ApoA1. Data are mean ± SEM. *Statistically significant difference between vehicle and T0901317-treated animals of the same genotype ($p \leq 0.05\%$). †Statistically significant difference between LXR+ and DKO with the same treatment ($p \leq 0.05\%$).

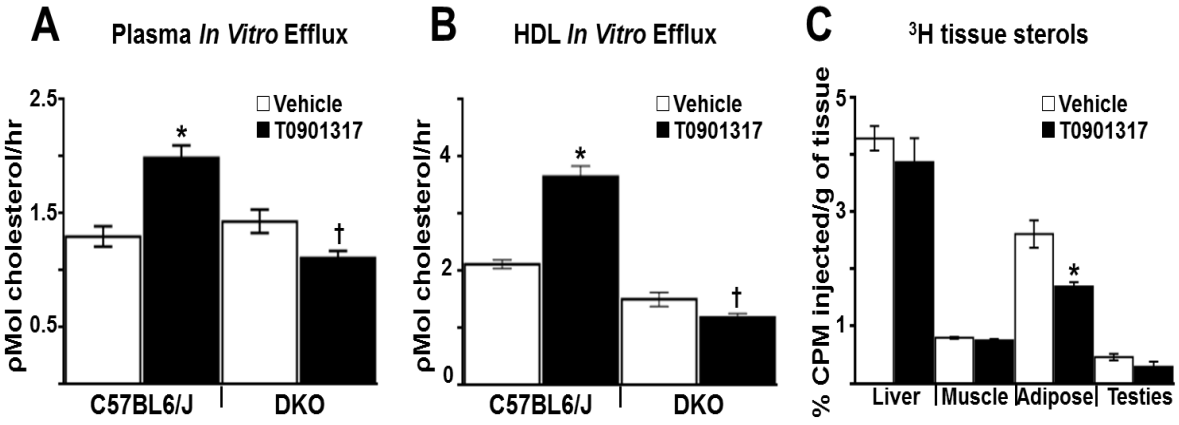


Figure 2.4 ***HDL function and adipose tissue drives LXR-dependent RCT.***

Figure 2.4 ***HDL function and adipose tissue drives LXR-dependent RCT.*** C57BL6/J and LXRα$^{-/-}$/LXRβ$^{-/-}$ (DKO) mice (n = 5-6/group) were treated for 5 days with vehicle or 10 mg/kgT0901317 and *In vitro* macrophage cholesterol efflux was measured as described in Materials and Methods using ^{3}H-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**) ^{3}H-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 ^{3}H 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 ($p \leq 0.05\%$). † Statistically significant difference between C57BL6/J and DKO with the same treatment ($p \leq 0.05\%$).

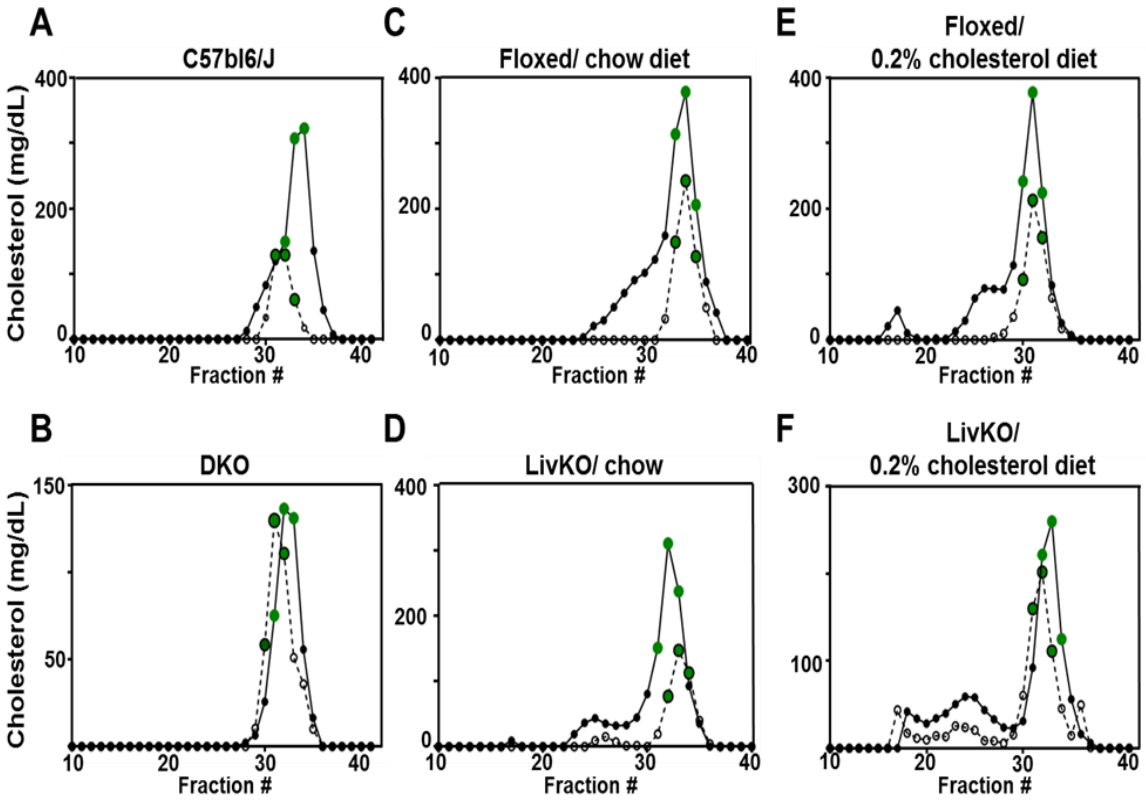


Figure 2.5 ***FPLC profiles.***

Figure 2.5 ***FPLC profiles.*** **A)** C57bl6/J; **B)** LXRα$^{-/-}$ LXRβ$^{-/-}$ (DKO); **C)** LXRα$^{fl/fl}$ albumin-CRE^{-} (Floxed) on chow diet; **D)** LXRα$^{fl/fl}$ albumin-CRE+ (LivKO) on chow diet; **E)** Floxed on 0.2% cholesterol diet for 4 weeks; and **F)** LivKO on 0.2% cholesterol diet for 4 weeks 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. (n=5-6/group) The three fractions containing the greatest amounts of cholesterol were pooled (green circles) and used in additional experiments.

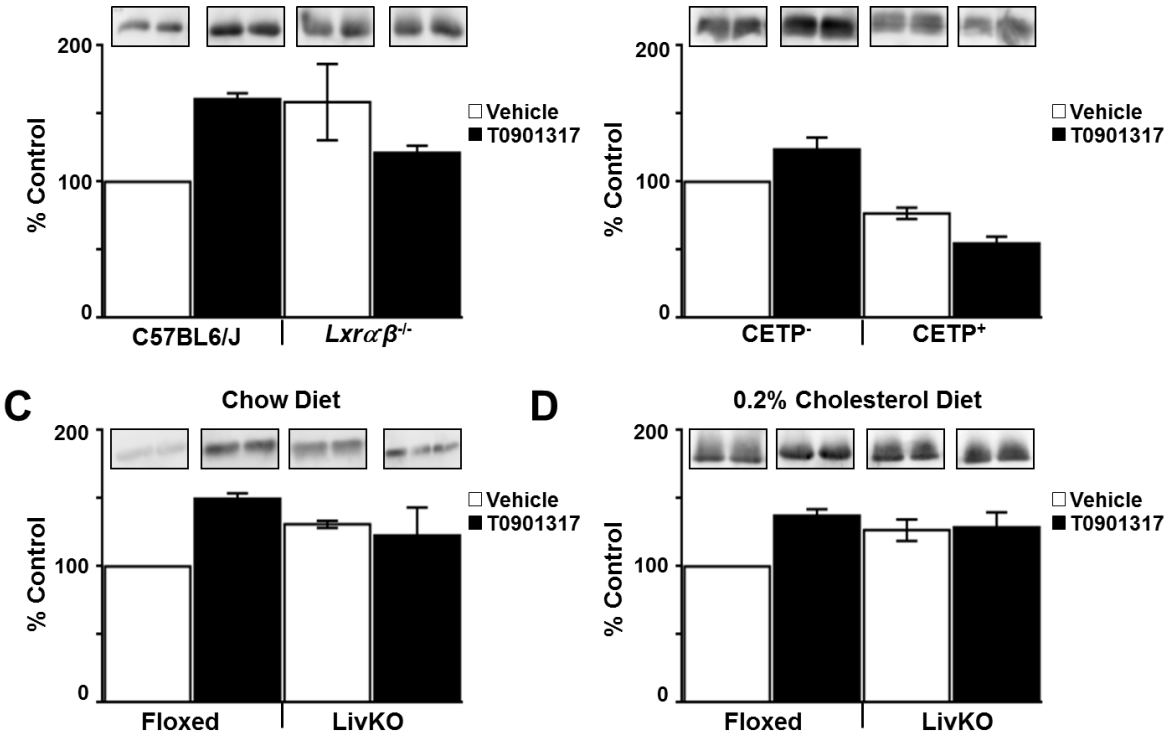


Figure 2.6 ***APOA1 protein levels in FPLC-purified HDL.***

Figure 2.6 ***APOA1 protein levels in FPLC-purified HDL.*** Pooled plasma from vehicle or T0901317-treated C57BL6/J, *Lxr*$\alpha^{-/-}$ *Lxr*$\beta^{-/-}$ (*Lxr*$\alpha^{-/-}\beta^{-/-}$), Floxed and LivKO animals were subjected to FPLC for lipoprotein analysis. **A-D**) Peak HDL fractions were pooled and APOA1 protein levels were measured by Western blot (n = 4-6/group). Quantification of APOA1 levels was carried out as described in Materials and Methods. Vehicle treated C57BL6/J, Floxed and CETP^{-} was set as 100%.

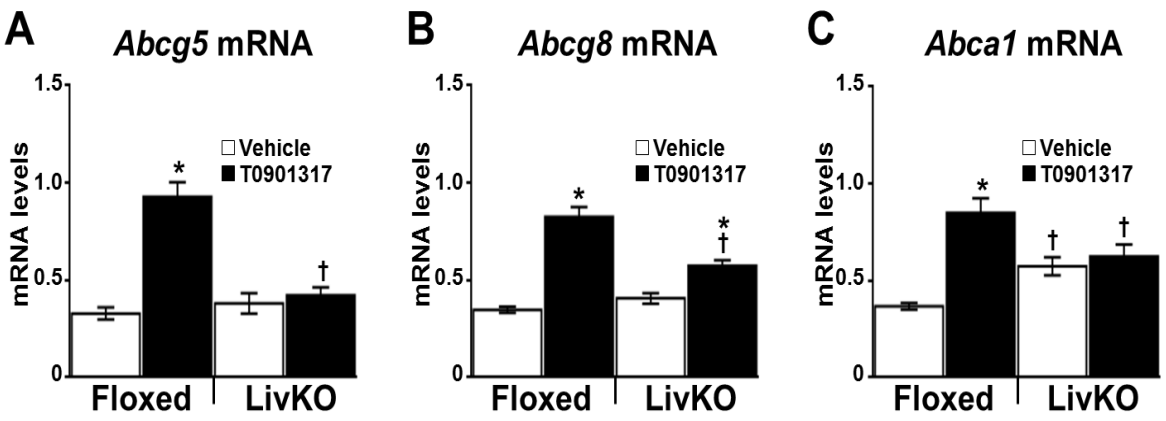


Figure 2.7 ***Hepatic ABC transporter expression in LivKO mice.***

Figure 2.7 ***Hepatic ABC transporter expression in LivKO mice.*** Total RNA was extracted from liver tissue at the completion of the *in vivo* RCT experiment and mRNA levels of ABCG5 **(A),** ABCG8 (**B**) and ABCA1 (**C**) were measured by quantitative real-time PCR. Data are mean ± SEM. *Statistically significant difference between vehicle and T0901317-treated animals of the same genotype ($p \leq 0.05\%$). †Statistically significant difference between Floxed and LivKO with the same treatment ($p \leq 0.05\%$).

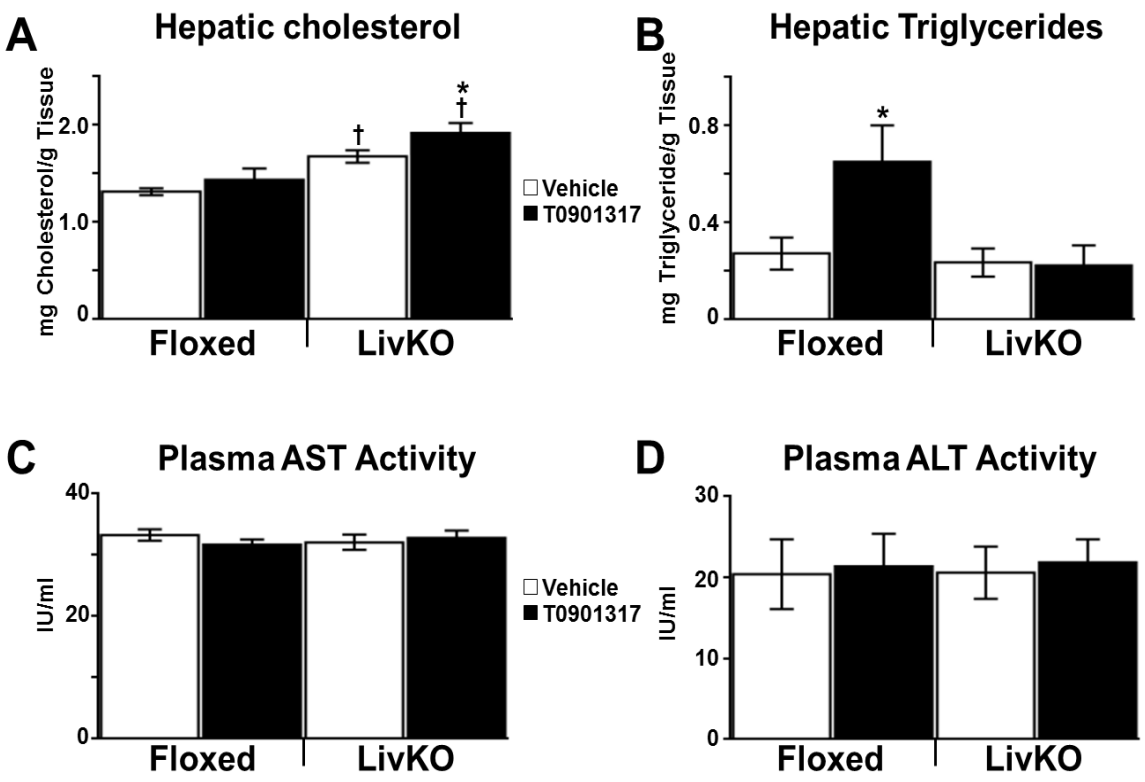


Figure 2.8 ***Effect of 0.2% cholesterol diet on hepatic lipids and liver enzymes***.

Figure 2.8 ***Effect of 0.2% cholesterol diet on hepatic lipids and liver enzymes***. At the completion of the in vivo RCT study hepatic cholesterol, hepatic triglycerides, AST activity and ALT activity were determined as described in Materials and Methods. Data are mean ± SEM, (n=5-6/group). *Statistically significant difference between vehicle and T0901317-treated animals of the same genotype ($p \leq 0.05\%$). †Statistically significant difference between Floxed and LivKO with the same treatment ($p \leq 0.05\%$).

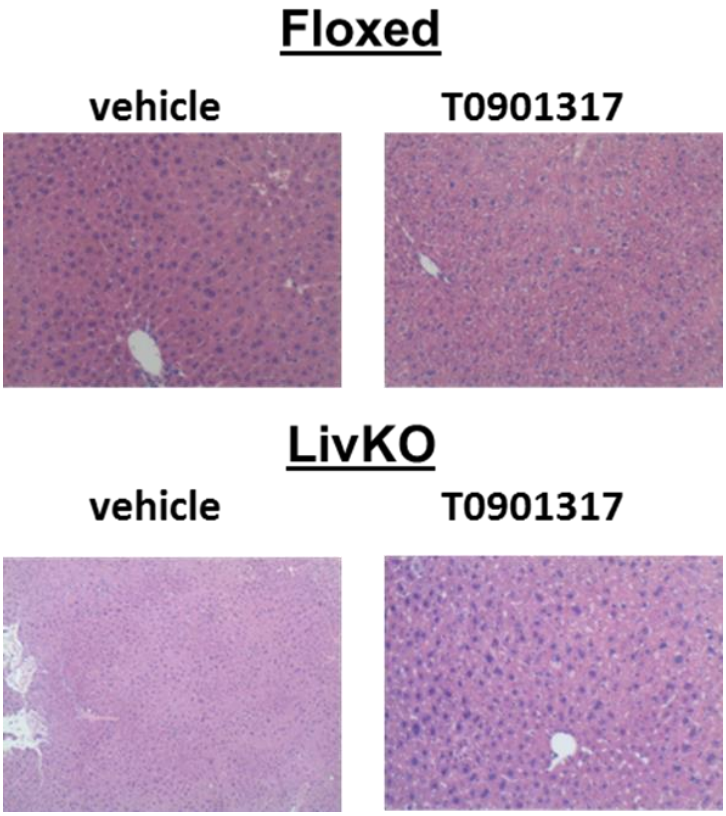


Figure 2.9 ***Liver histology in 0.2% cholesterol diet fed LivKO mice.***

Figure 2.9 ***Liver histology in 0.2% cholesterol diet fed LivKO mice*****.** Following 4 weeks of 0.2% cholesterol diet feeding, LivKO and Floxed controls were treated with vehicle or 10 mg/kg T0901317 for 5 days. Whole livers were isolated and snap frozen in liquid nitrogen. Frozen livers were then sectioned and H and E stained by University of Virginia Tissue Histology core facility.

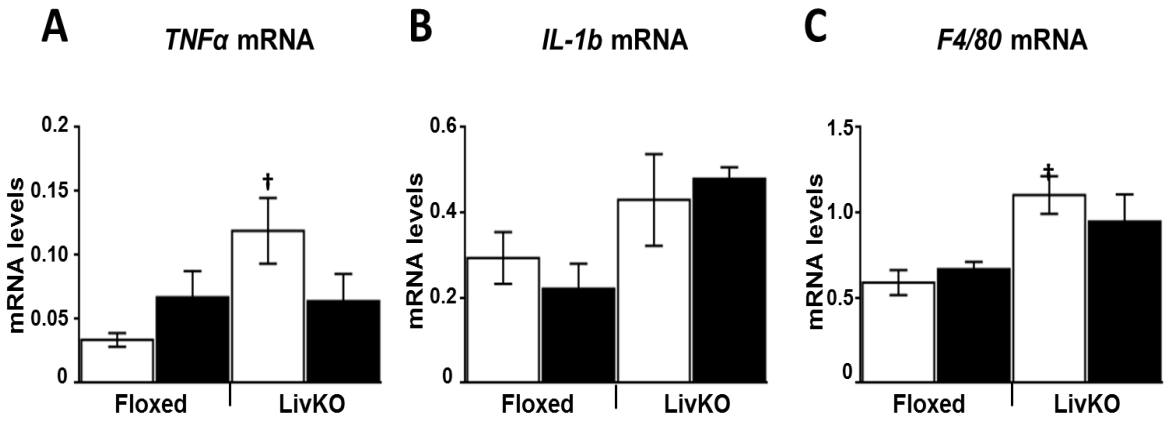
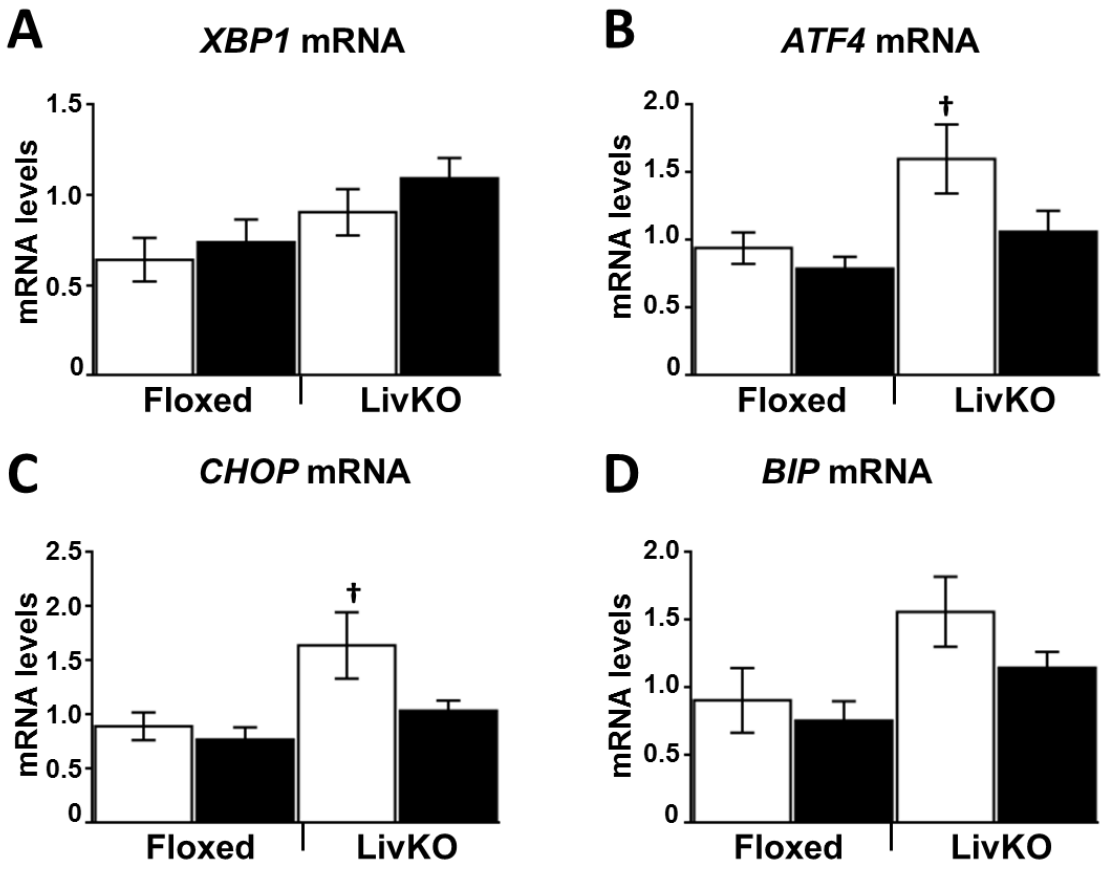


Figure 2.10 ***Inflammatory gene expression in 0.2% cholesterol diet fed LivKO mice.***

Figure 2.10 ***Inflammatory gene expression in 0.2% cholesterol diet fed LivKO mice.*** Total RNA was isolated from livers at the completion of *in vivo* RCT studies and mRNA levels were measured by quantitative real-time PCR as described in Materials and Methods. Data is mean ± SEM, (n=5-6/group). [†]Statistically significant difference between Floxed and LivKO with the same treatment ($p \leq 0.05\%$).



Supplemental Figure 2.11 ***Gene expression of endoplasmic reticulum stress in 0.2% cholesterol diet fed LivKO mice.***

Figure 2.11 ***Gene expression of endoplasmic reticulum stress in 0.2% cholesterol diet fed LivKO mice.*** Total RNA was isolated from livers at the completion of *in vivo* RCT studies and mRNA levels were measured by quantitative real-time PCR as described in Materials and Methods. Data is mean ± SEM, (n=5-6/group). [†]Statistically significant difference between Floxed and LivKO with the same treatment ($p \leq 0.05\%$).

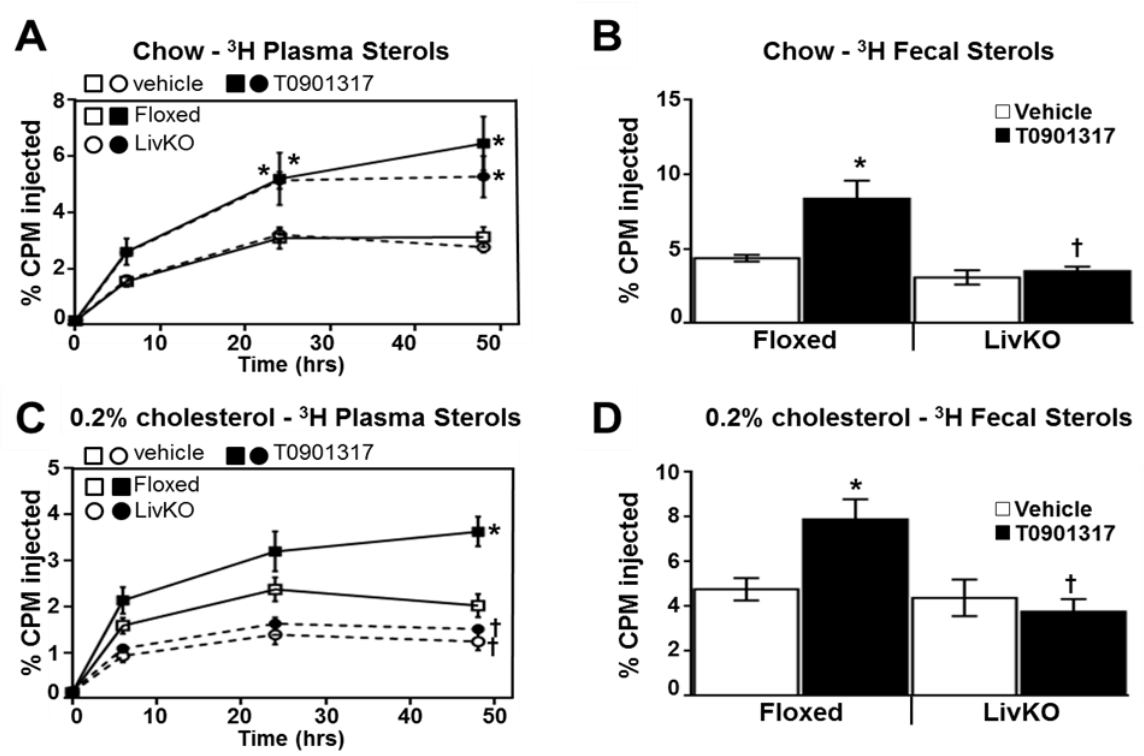


Figure 2.12 In vivo *RCT in chow and 0.2% cholesterol diet fed LivKO mice*.

Figure 2.12 In vivo *RCT in chow and 0.2% cholesterol diet fed LivKO mice*. ^{3}H-cholesterol and acetylated LDL-loaded C57BL6/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/group) prior to BMM injection, and the amount of ^{3}H sterol in plasma (**A** and **C**) and feces (**B** and **D**) was determined as described in the Materials and Methods. For the 0.2% diet experiment animals were switched from chow to 0.2% diet at 8 weeks of age and were on diet for 4 weeks prior to the start of the experiment. Mice continued to receive vehicle or T0901317 treatment for the duration of the experiment. Data are mean ± SEM. *Statistically significant difference between vehicle and T0901317-treated animals of the same genotype ($p \leq 0.05\%$). †Statistically significant difference between Floxed and LivKO mice with the same treatment ($p \leq 0.05\%$).

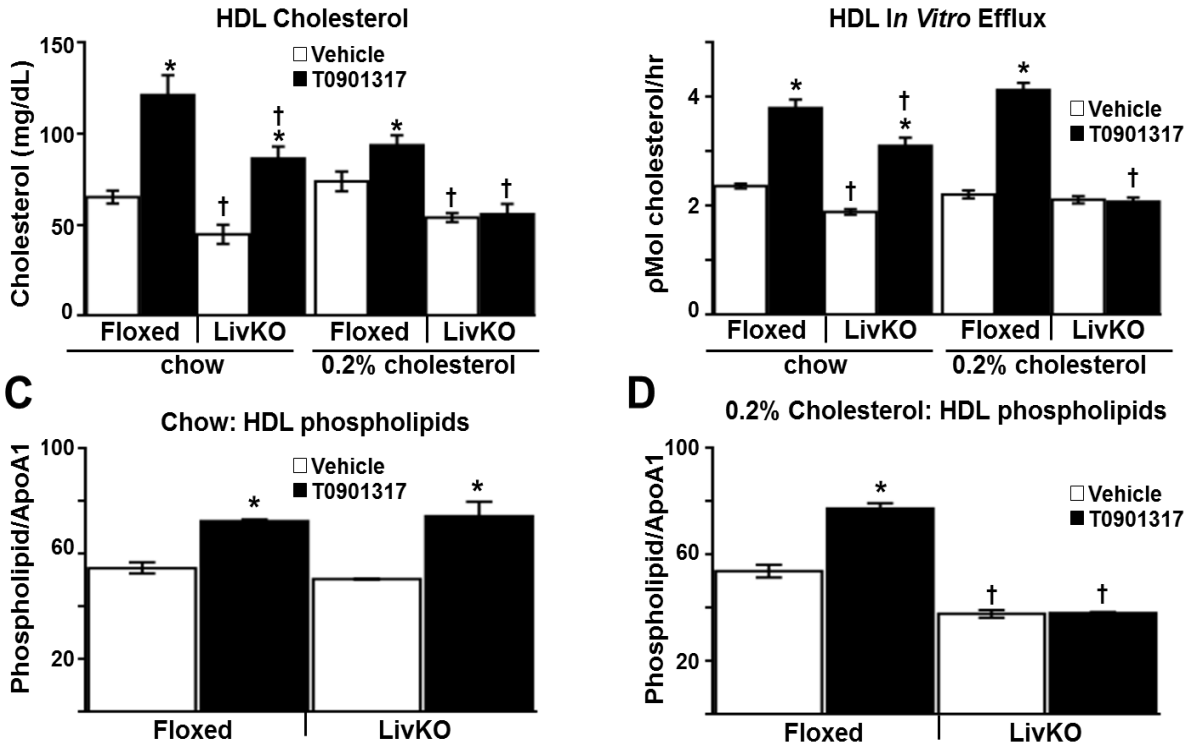


Figure 2.13 ***LXR agonist dependent changes in HDL mass and function in LivKO mice.***

Figure 2.13 ***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/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 ^{3}H-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/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 Elisa. Data are mean ± SEM. *Statistically significant difference between vehicle and T0901317-treated animals of the same genotype ($p \leq 0.05\%$). †Statistically significant difference between Floxed and LivKO mice with the same treatment and diet ($p \leq 0.05\%$).

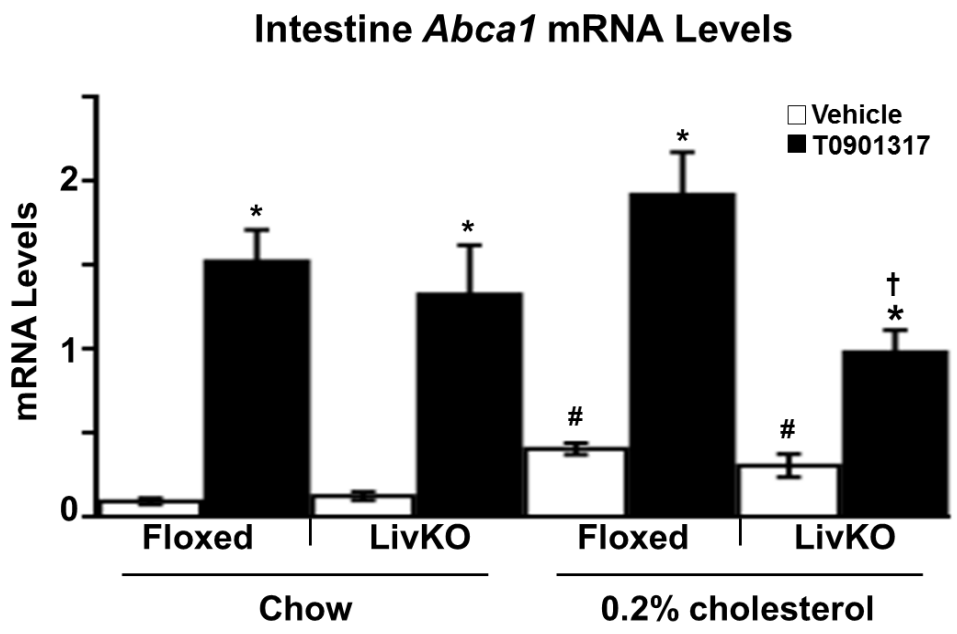


Figure 2.14 ***Intestine ABCA1 levels in chow and 0.2% cholesterol diet fed LivKO mice.***

Figure 2.14 Intestine ABCA1 levels in chow and 0.2% cholesterol diet fed LivKO mice. Total RNA was isolated from intestines at the completion of *in vivo* RCT studies and ABCA1 mRNA levels were measured by quantitative real-time PCR as described in Materials and Methods. Data is mean ± SEM, (n=5-6/group). *Statistically significant difference between vehicle and T0901317-treated animals of the same genotype and diet conditions ($p \leq 0.05\%$). †Statistically significant difference between Floxed and LivKO with the same treatment ($p \leq 0.05\%$). #Statistically significant difference between chow and 0.2% cholesterol diet fed mice of the same genotype and treatment ($p \leq 0.05\%$).

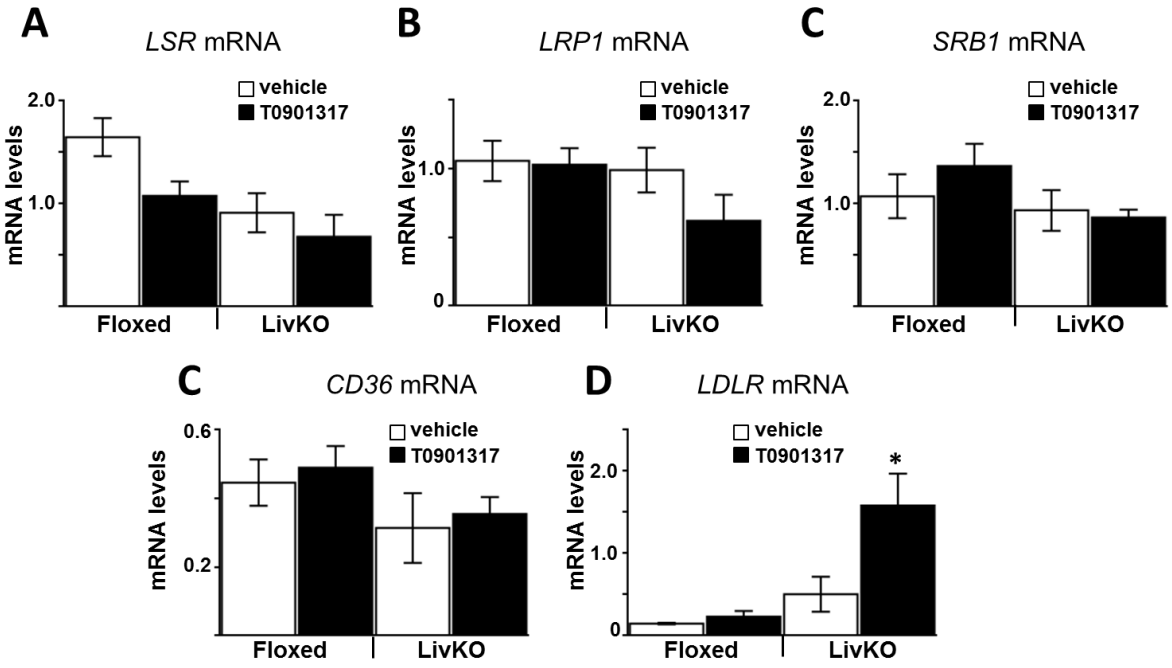


Figure 2.15 ***Gene expression analysis of lipoprotein receptors in LivKO mice.***

Figure 2.15 ***Gene expression analysis of lipoprotein receptors in LivKO mice.*** Total RNA was isolated from livers at the completion of *in vivo* RCT studies and mRNA levels were measured by quantitative real-time PCR as described in Materials and Methods. Data is mean ± SEM, (n=5-6/group). *Statistically significant difference between vehicle and T0901317-treated animals of the same genotype ($p \leq 0.05\%$).

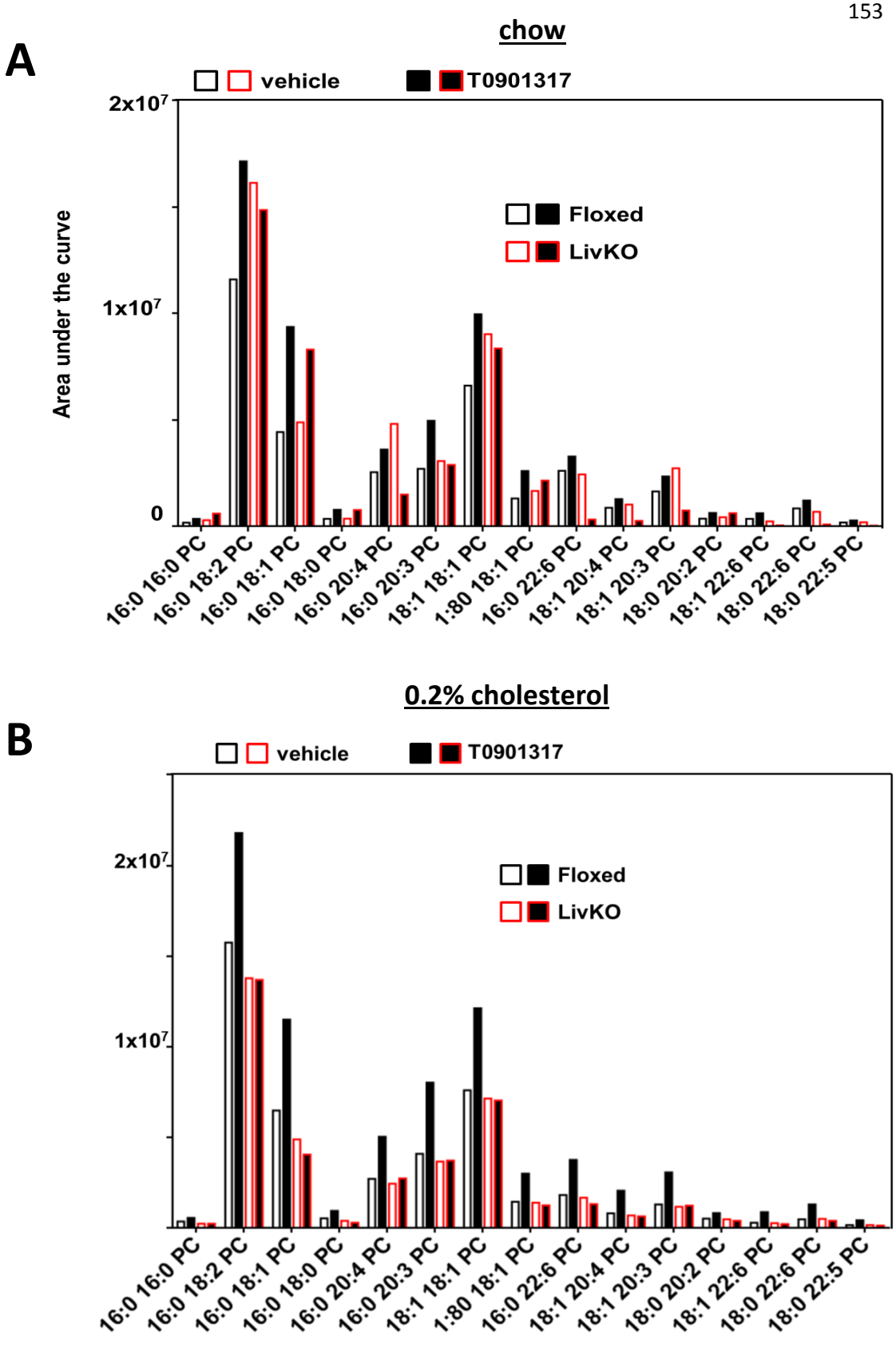


Figure 2.16 ***phospholipid composition of HDL particles from LivKO mice.***

Figure 2.16 ***phospholipid analysis of HDL particles from LivKO mice.*** Animals on chow or 4 weeks of 0.2% cholesterol diet were treated with vehicle or 10 mg/kg T0901317 for 5 days and pooled plasma was fractionated by FPLC. Phospholipid species were identified from FPLC-purified HDL by mass spectrometry as described in Materials and Methods and normalized by ApoA1 levels as determined by Western blot.

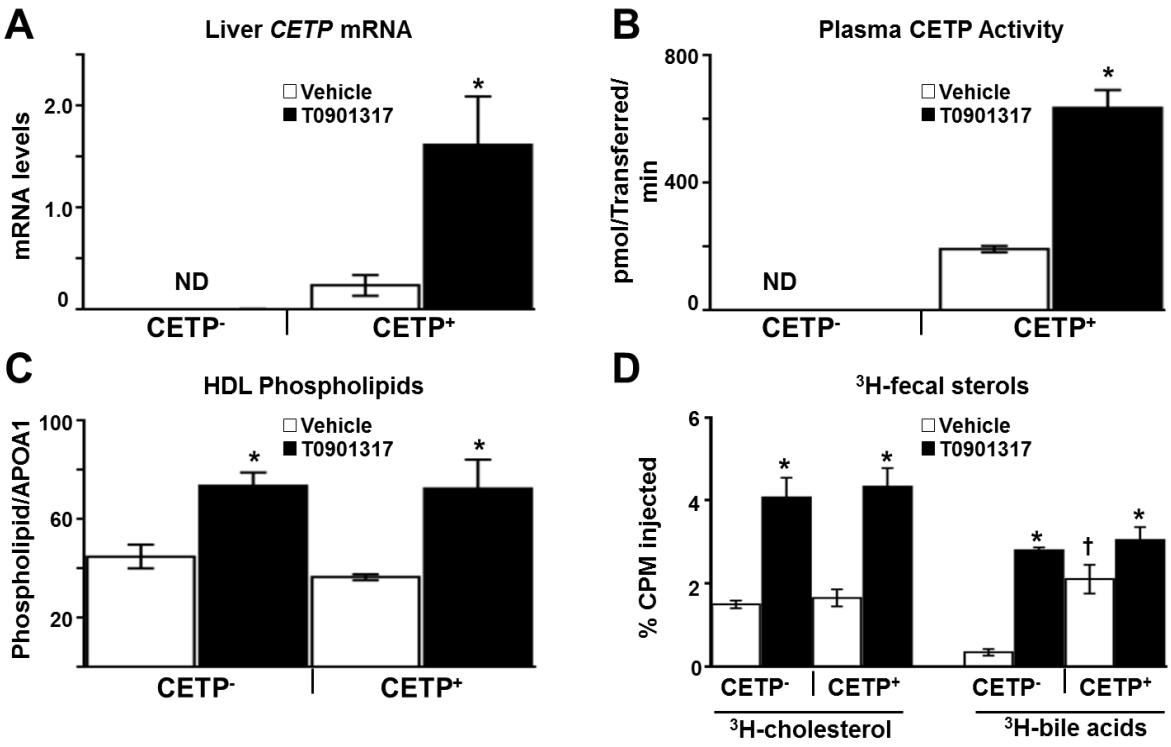


Figure 2.17 ***LXR agonists increase CETP activity, HDL-phospholipids and fecal ^{3}H-sterols in CETP transgenic mice.***

Figure 2.17 ***LXR agonists increase CETP activity, HDL-phospholipids and fecal ^{3}H-sterols in CETP transgenic mice.*** CETP^{-} and CETP^{+} mice were treated vehicle or 10 mg/kg T0901317 for 5 days (n=5/group). **A**) Total RNA was isolated from liver tissue and the mRNA levels of *CETP* was measured by quantitative real-time PCR as described in Materials and Methods. **B**) CETP activity in plasma was determined by fluorometric assay as described in Materials and Methods. **C**)Total phospholipids in FPLC purified HDL from CETP^{-} and CETP^{+} mice treated for 5 days with vehicle or T0901317 (10 mpk). HDL phospholipid levels were normalized by HDL APOA1 protein levels as determined by Elisa. **D**) At completion of the *in vivo* RCT experiment fecal sterols were extracted and the amount of ^{3}H-cholesterol and ^{3}H-bile acids were determined as described in Materials and Methods. Data are mean ± SEM. *Statistically significant difference between vehicle and T0901317-treated animals of the same genotype($p \leq 0.05\%$). †Statistically significant difference between CETP^{-} and CETP^{+} with the same treatment ($p \leq 0.05\%$). ND = not detected.

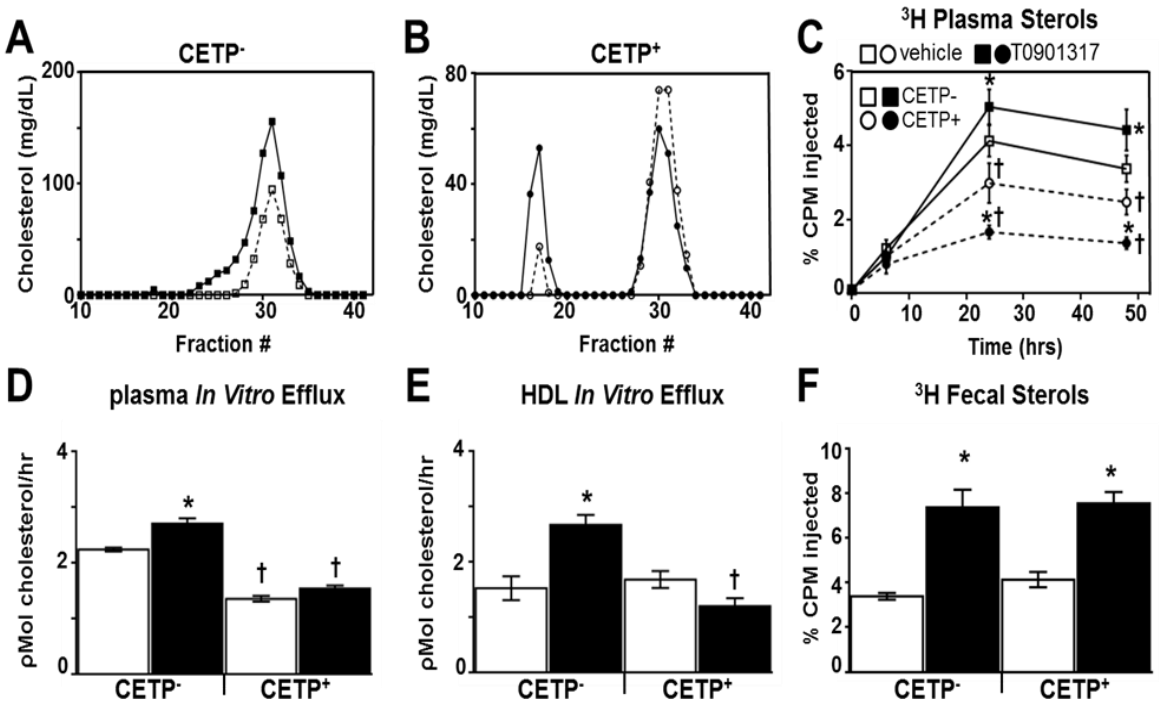


Figure 2.18 ***CETP inhibits LXR agonist-dependent RCT.***

Figure 2.18 ***CETP inhibits LXR agonist-dependent RCT.*** $CETP^-$ (**A**) and $CETP^+$ (**B**) mice (n=6/group) were treated with vehicle or T0901317 (10 mpk), plasma was pooled, subjected to FPLC and the cholesterol content of each fraction was measured as described in Materials and Methods. ^{3}H-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 ^{3}H sterol in plasma (**C**) and feces (**F**) were 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 ($p \leq 0.05\%$). †Statistically significant difference between $CETP^-$ and $CETP^+$ with the same treatment ($p \leq 0.05\%$).

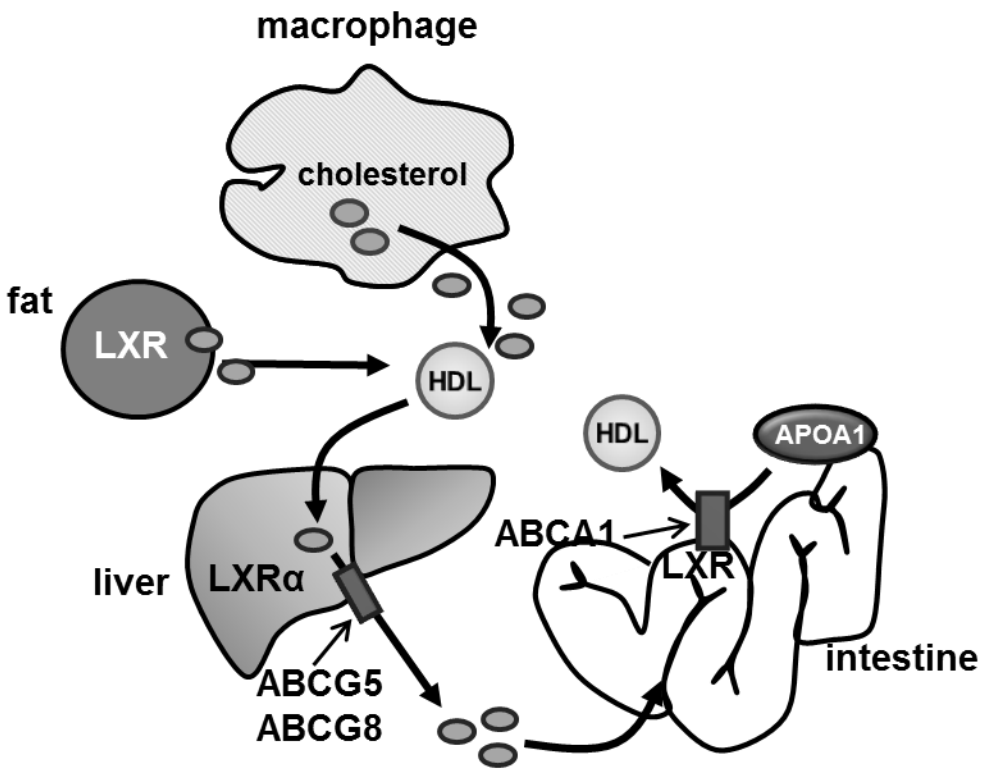


Figure 2.19 ***Model for LXR-regulated RCT.***

Chapter 3: Liver LXRα expression is crucial for whole body cholesterol homeostasis and reverse cholesterol transport in mice

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Zhang and colleagues in the Mangelsdorf Laboratory at the University of Texas Southwestern Medical Center generated the first conditional LXR knockout animal by selectively eliminating LXRα from hepatocytes and this group was responsible for the initial characterization of these albumin-CRE^{+} LXRα$^{fl/fl}$ mice. Interested in the role of liver LXRα in atherosclerosis and the protective effects of LXR agonists, Dr.Schulman received the LivKO mice from the Mangelsdorf group and with Jerry Angidsen's assistance crossed them into the Ldlr$^{-/-}$ background prior to the start of my graduate studies. The first atherosclerosis study with the Ldlr$^{-/-}$ /LivKO was undertaken by Dr. Schulman with Jerry's technical assistance. The finding that the Ldlr$^{-/-}$/LivKO animals had increased atherosclerosis, led me to investigate the role for liver LXRa in atherosclerosis and reverse cholesterol transport. The outcome of this work, which was undertaken during my first two years in the laboratory, resulted in a co-first authorship on the manuscript which appeared in the Journal of Clinical Investigations in April 2012.

3.1 ABSTRACT

Liver X receptors (LXRα and LXRβ) are important regulators of cholesterol and lipid metabolism and their activation has been shown to inhibit cardiovascular disease and reduce atherosclerosis in animal models. These previous studies have stimulated interest in the therapeutic potential of small molecules targeting LXRs; however, the finding that agonists also promote hepatic lipogenesis has led to the idea that hepatic LXR activity is undesirable from a therapeutic perspective. In this report we utilized gene targeting to create the first conditional LXR knockout by selectively deleting LXRα in hepatocytes. Liver-specific deletion of LXRα substantially decreased reverse cholesterol transport, cholesterol catabolism and excretion, revealing the essential importance of hepatic LXRα for whole body cholesterol homeostasis. Additionally, in a pro-atherogenic background liver-specific deletion of LXRα significantly increased atherosclerosis, uncovering an important function for hepatic LXR activity in limiting cardiovascular disease. Nevertheless, LXR agonists still elicited anti-atherogenic activity in the absence of hepatic LXRα indicating that the ability of agonists to reduce cardiovascular disease does not require an increase in cholesterol excretion. Furthermore these observations suggest that therapeutic strategies which bypass the liver or limit the activation of hepatic LXRs should still be beneficial for the treatment of cardiovascular disease.

3.2 INTRODUCTION

The precise regulation of cholesterol metabolism is essential and it is well known that elevated levels of cholesterol in the blood are a major cause of cardiovascular disease [674]. Studies using global genetic knockouts and synthetic agonists have defined important roles for the liver X receptors, LXRα (NR1H3) and LXRβ (NR1H2), in the control of cholesterol metabolism [640]. LXRs are members of the nuclear hormone receptor superfamily of ligand activated transcription factors and treatment of animals with LXR agonists results in changes in gene expression promoting the efflux of cholesterol from peripheral cells such as macrophages, the excretion of cholesterol from the liver and the inhibition of cholesterol absorption in the intestine [640]. Importantly, the endogenous ligands for LXRs are oxidized forms of cholesterol (oxysterols) [641, 675] that increase coordinately with intracellular cholesterol levels thus allowing these receptors to function as sensors to maintain cholesterol at appropriate levels throughout the body.

At the molecular level, LXRs control cholesterol efflux by regulating expression of the genes encoding the ATP binding cassette (ABC) transporters ABCA1 and ABCG1 [640]. Up-regulation of ABCA1 and ABCG1 results in increased transfer of intracellular cholesterol to high density lipoprotein particles (HDL) and genome wide association studies have linked both transporters to HDL cholesterol levels in humans [642, 643]. Mutations in the human *ABCA1* gene result in Tangier disease and Tangier patients characteristically present with little or no HDL, massive accumulation of cholesterol in macrophages found lodged in lymph tissue and they exhibit an increased risk for atherosclerosis [638, 644, 645]. The accumulation of oxidized and other modified forms of cholesterol by macrophages present in blood vessel walls is a critical event in the pathogenesis of atherosclerosis [676] and the ability of LXR agonists to enhance

macrophage cholesterol efflux has stimulated great interest in the therapeutic potential of these compounds [677]. Activation of LXRs also regulate expression of ABCG5 and ABCG8, two half transporters that dimerize to create an additional cholesterol transporter [573, 646]. Expression of ABCG5/ABCG8 is largely restricted to the liver and intestine [34] where these proteins function to promote the excretion of cholesterol (liver) and limit cholesterol absorption (intestine). Thus by mobilizing cholesterol from the periphery, promoting hepatic excretion and limiting absorption, activation of LXRs results in a net loss of cholesterol. This process of trafficking cholesterol to HDL and ultimately out of the body has been termed reverse cholesterol transport (RCT) [638, 645]. Importantly LXR agonists decrease atherosclerosis in animal models and it has been suggested that enhanced RCT plays an important role in this activity [548, 549, 552, 628].

In spite of many potential benefits on cholesterol metabolism, enthusiasm for the therapeutic value of LXR agonists has been tempered by the observation that LXR activation stimulates hepatic lipogenesis by increasing expression of sterol regulatory element binding protein-1c (SREPB-1c), a master transcriptional regulator of fatty acid and triglyceride synthesis [524, 583]. Along with *Srebp1c*, LXRs regulate either directly or indirectly the genes encoding a number of other proteins involved in fatty acid synthesis [586, 678] and treatment with LXR agonists can result in dramatic increases in hepatic and plasma triglycerides [524, 583]. Additionally, at least one class of synthetic LXR ligands has been shown to elevate plasma low density lipoprotein (LDL) cholesterol levels in non-human primates [597]. Genetic studies have defined the LXRα subtype as the major regulator of hepatic lipogenesis in response to LXR agonists [628, 679]. The simple idea of creating LXRβ-specific ligands to bypass the undesirable effects on lipogenesis, however,

has been challenging because the ligand binding pockets of the two LXR subtypes differ by only a single amino acid [680, 681]. Studies in LDL receptor and apolipoprotein E (*Apoe*) knockout mice have also demonstrated that it is the LXRα subtype which plays the dominant role in limiting diet-induced cardiovascular disease [558, 628]. These observations have led to the suggestion that LXR agonists that bypass the liver, or even function as antagonists in the liver, would have ideal therapeutic profiles [677, 682, 683]. To address the therapeutic potential of liver LXR activity we have used gene targeting technology to create the first conditional LXR knockout mouse line by selectively deleting LXRα in hepatocytes. Characterization of these animals demonstrates the essential, physiologic importance of hepatic LXRα to whole body cholesterol homeostasis while at the same time revealing the pharmacologic utility of bypassing hepatic LXR activity as a therapeutic strategy for treating cardiovascular disease.

3.3 RESULTS

Summary of initial characterization of Liver LXRα deficient animals.

Below is a summary of the work carried out in the Mangelsdorf Lab prior to our acquisition of the liver-specific LXRα knockout mice.

Generation of liver-specific LXRα knockout mice LXRα floxed mice were crossed with albumin-Cre mice to generate hepatocyte-specific knockout of LXRα (*Lxrα*$^{fl/fl}$ albumin-CRE^{+}, referred to as LivKO) and their floxed littermate controls (*Lxrα*$^{fl/fl}$ albumin-CRE^{-}, referred to as Floxed). LXRα mRNA was reduced by more than 95% in livers of LivKO mice while the expression of LXRβ did not change. There was no change in LXRα or LXRβ expression in any other tissue. Feeding LivKO animals a diet containing 2% cholesterol for 30 days resulted in significant hepatic cholesterol accumulation and an increased liver to body weight ratio, demonstrating that liver LXRα activity is responsible for this phenotype that is observed in the global LXRα knockout under the same condition[678].

Hepatic LXRα regulates lipid metabolism. Treatment with LXR agonists has been shown to increase triglyceride levels, promote cholesterol excretion, and elevate plasma HDL[640]. Following 2 days of agonist treatment, an increase in plasma triglycerides was observed in control mice that was attenuated in the LivKO animals. Analysis of hepatic gene expression indicated that agonist-dependent increase in *Srebp1*, as well as fatty acid synthase (*Fas*) and stearoyl CoA desaturase 1 (*Scd1*), was reduced in LivKO. Previous studies with global knockouts demonstrated that LXRα is a major regulator of hepatic SREBP-1c expression and triglyceride levels[559, 679]. Taken together, the lipid measurements and gene expression analysis of LivKO mice support this conclusion and

further indicate that hepatic LXRα activity is responsible for most of the lipogenic activity of LXR agonists.

Regulation of *Abcg5* and *Abcg8* in the liver and intestine has been proposed to account for the ability of LXR agonists to stimulate the biliary secretion of cholesterol and decrease intestinal absorption, resulting in increased neutral sterol loss in the feces[575, 646, 684]. Recent studies, however, have described a biliary-independent trans-intestinal pathway for cholesterol excretion that can be stimulated by LXR activity[577, 685, 686]. In the absence of liver LXRα activity, the ability of LXR agonists to increase biliary cholesterol was abolished and fecal cholesterol excretion was decreased. The loss of agonist-stimulated effects in LivKO mice coincided with a failure to increase *Abcg5* and *Abcg8* in the liver, while *Abcg5* and *Abcg8* expression in the intestine was unaffected. Therefore, hepatic LXRα activity is required for the majority of the LXR agonist-dependent increase in cholesterol excretion. Moreover, these results support the notion that hepatic, and not intestinal, ABCG5/G8 is required for LXR-dependent effects on cholesterol excretion.

The regulation of bile acid synthesis by LXR has also been suggested to contribute to the ability of LXR agonists to dispose of cholesterol[520, 678]. As expected, the ability of LXR agonists to increase the expression of Cyp7a1, which encoded the rate-limiting enzyme in the conversion of cholesterol to bile acids, was absent in LivKO mice. Nevertheless, there was no effect of LXR agonist treatment on fecal or biliary bile acids or on the bile acid pool size between LivKO and floxed animals. These results support previous studies showing that LXR agonists have no effect on fecal bile salt excretion despite the increase in *Cyp7a1* expression, which has been suggested to be due to the fast reabsorption and recycling of bile acids. Along with regulation of *Cyp7a1*, treatment of

control animals with an LXR agonist resulted in decreased expression of *Cyp8b1*, the gene encoding sterol 12α-hydroxylase, and this effect was lost in the LivKO mice. Sterol 12α-hydroxylase sits at a branch point in the bile acid synthetic pathway, and its enzymatic activity is required for the synthesis of cholic acid (CA). The parallel arm in the pathway leads to synthesis of muricholic acid (MCA) in mice[687]. Consistent with the gene expression data, treatment with T0901317 decreased the ratio of CA to MCA in control mice but not in LivKO. Importantly, individual bile acids differ in their ability to promote intestinal cholesterol absorption, and MCA, among all bile acids tested, promotes the lowest amount of cholesterol absorption, while CA promotes the greatest amount[688]. Thus, the agonist-dependent change in bile acid composition should contribute to the ability of LXR ligands to reduce cholesterol absorption. Consistent with this hypothesis, the ability of T0901317 to decrease fractional cholesterol absorption was also significantly attenuated in LivKO mice.

Hepatic LXRα is not required for the agonist-dependent HDL cholesterol regulation. The liver is considered the major site of HDL production[580, 689], and treatment of chow-fed mice with LXR agonists is known to increase HDL cholesterol levels[524, 583]. After treatment with T0901317 for 8 days, a significant increase in plasma cholesterol as observed in both LivKO mice and control mice, although the levels in T0901317-treated LivKO mice were approximately 15% lower than in control animals. Fractionation of lipoprotein particles by fast protein liquid chromatography (FPLC) indicated that the increase primarily resided in the HDL fraction. Thus, LXRα activity in the liver is not required for LXR agonist stimulated increases in HDL cholesterol levels in the plasma. LXR agonists have been shown to increase the presence of large HDL particles[573, 577, 589],

an effect that was attenuated in T0901317-treated LivKO mice, suggesting that hepatic LXRα can play a role in modulating HDL size. Consistent with these results the expression of several apolipoproteins was altered in LivKO mice. ABCA1 is required for the biogenesis of HDL, and studies with ABCA1-knockout mice indicate that both the liver and intestine contribute to HDL production[580, 689]. Induction of *Abca1* mRNA by T0901317 was lost in the livers of LivKO animals, while the intestinal *Abca1* levels were not different from those in control mice. The strong LXR agonist-dependent induction of Abca1 in the intestine suggests that this organ serves as a major site for LXR-dependent HDL cholesterol increases, a conclusion supported by earlier studies using tissue-specific knockouts of *Abca1* and intestine-specific overexpression of LXRα[579, 662].

Deletion of hepatic LXRα increases atherosclerosis. LXR agonists decrease atherosclerosis in animal models of cardiovascular disease [548, 549, 552, 628] and global deletion of LXRα increases atherosclerosis in either LDL receptor (*Ldlr*) knockout or *ApoE* knockout genetic backgrounds [558, 628]. Using a series of bone marrow transplantations we have previously demonstrated that cells derived from the hematopoietic system comprise an important site of LXR-dependent anti-atherogenic activity [549, 628]. These studies, however, also indicated important anti-atherogenic functions for LXRα in a site(s) that is not derived from bone marrow cells [628]. To determine the impact of liver LXRα activity on atherosclerosis, the liver specific knockout was introduced into the $Ldlr^{-/-}$ background. The resulting double knockouts ($Ldlr^{-/-}/Lxr\alpha^{fl/fl}$ albumin-CRE^{+}; i.e. $Ldlr^{-/-}$/LivKO) and littermate controls ($Ldlr^{-/-}/Lxr\alpha^{fl/fl}$ albumin-CRE^{-}; i.e. $Ldlr^{-/-}$/floxed) were placed on a high fat/high cholesterol Western diet for 20 weeks in the absence or presence of the LXR agonist T0901317. By 4

weeks on diet *Ldlr*$^{-/-}$/LivKO animals had reduced plasma triglycerides and cholesterol compared to controls and the effect of T0901317 on plasma lipid levels was lost in the *Ldlr*$^{-/-}$/LivKO mice (Figure 3.1A-D). Consistent with other studies in hyperlipidemic mouse models [548, 549, 552, 558, 628], treatment with LXR agonist had little or no effect on HDL cholesterol levels in either *Ldlr*$^{-/-}$/floxed or *Ldlr*$^{-/-}$/LivKO animals (Figure 3.1E-F). As expected, hepatic cholesterol was substantially increased in *Ldlr*$^{-/-}$/LivKO animals at the conclusion of the experiment (Figure 3.2B).

When atherosclerosis was quantitated by *en face* analysis of dissected aortas or by serial sections of the aortic root, a significant increase in lesion area was detected in *Ldlr*$^{-/-}$/LivKO mice compared to controls (Figure 3.3 and Figure 3.4). Immunostaining with the macrophage-specific antibody MOMA-2 indicated increased macrophage content in *Ldlr*$^{-/-}$/LivKO root sections. Collagen staining, a measure of plaque stability, was roughly similar (Figure 3.5). A similar increase in atherosclerosis was also observed in *Ldlr*$^{-/-}$/LivKO mice after 10 weeks on Western diet (Figure 3.6). Thus, LXRα activity in the liver plays an essential role in limiting cardiovascular disease in the background of the *Ldlr*-null animal. Importantly, T0901317 was still able to significantly reduce atherosclerosis in *Ldlr*$^{-/-}$/LivKO mice (Figure 3.3, Figures 3.4 and 3.6), indicating that liver LXRα activity is not required for the pharmacological anti-atherogenic activity of LXR agonists. The magnitude of the agonist-dependent decrease in *Ldlr*$^{-/-}$/LivKO mice was similar to observed in *Ldlr*$^{-/-}$/floxed controls (30-40%) suggesting that the full therapeutic effect of LXR agonists can be manifested in the absence of liver LXRα.

***Lipoprotein particle number, size and function in LivKO mice*.** We noted that *Ldlr*$^{-/-}$/LivKO mice have relatively high plasma cholesterol levels while their plasma

triglyceride levels are approximately 5 times less than *Ldlr*$^{-/-}$/floxed controls (Figure 3.1A-D). This large difference in plasma triglycerides suggested the possibility that the number and/or size of the lipoprotein particles produced in *Ldlr*$^{-/-}$/LivKO mice may be altered in a way that influences atherogenesis. To address this possibility we used nuclear magnetic resonance (NMR) spectroscopy to examine lipoprotein particle number and size [690]. The high triglyceride levels in T0901317 treated *Ldlr*$^{-/-}$/floxed mice precluded analysis of the effect of agonist treatment on particle number and size by NMR so we restricted this analysis to vehicle treated animals that had been on Western diet for 10 weeks. As expected, the number of VLDL particles is decreased in *Ldlr*$^{-/-}$/LivKO mice (Table 3.1) consistent with an important role for hepatic LXRα in triglyceride synthesis. In contrast, while both *Ldlr*$^{-/-}$/floxed and *Ldlr*$^{-/-}$/LivKO animals have similar numbers of LDL particles there is a dramatic change in particle size with almost 50% of the *Ldlr*$^{-/-}$/LivKO particles having diameters less than 21 nM (Table 3.1, small LDL). We note that the high percentage of relatively large LDL particles (diameter $\geq$ 23 nM) measured in *Ldlr*$^{-/-}$/floxed mice is consistent with previous studies in hyperlipidemic mice [691]. Although there is a clear difference in size between LDL particles of the two strains we did not detect a difference in cholesterol accumulation when bone marrow derived macrophages were cultured *in vitro* in the presence of plasma or FPLC purified apolipoprotein B containing lipoproteins from *Ldlr*$^{-/-}$/floxed or *Ldlr*$^{-/-}$/LivKO animals (data not shown).

Both FPLC (Figure 3.1E-F) and NMR (Table 3.1) indicate that there is no difference in HDL cholesterol levels between the *Ldlr*$^{-/-}$/floxed and *Ldlr*$^{-/-}$/LivKO animals. The *Ldlr*$^{-/-}$/LivKO mice, however, do have a 30% decrease in total HDL particle

number that is largely the result of a decrease in small HDL (particles with diameters less than 8.2 nm; Table 1). Interestingly, hepatic expression of the gene encoding phospholipid transfer protein (PLTP), a known LXR target gene [600], is significantly reduced in *Ldlr*$^{-/-}$/LivKO mice (Figure 3.7). PLTP has been shown to remodel HDL resulting in the production of small particles [692]. To examine if the change in particle number and size influences HDL function, we performed *in vitro* cholesterol efflux assays using ^{3}H-cholesterol loaded RAW 264.7 cells. Cholesterol efflux was significantly reduced when *Ldlr*$^{-/-}$/LivKO (Figure 3.8A) or FPLC-purified HDL (Figure 3.8B) was used as the source of cholesterol acceptors. We could not examine the efflux potential of plasma from T0901317 treated mice because the high concentrations of agonist in these plasma samples significantly increased the expression of genes that promote RCT in the cholesterol loaded RAW 264.7 cells. We did not, however, consistently detect differences in macrophage cholesterol efflux when FPLC purified HDL particles from vehicle and T0901317 treated mice were used as cholesterol acceptors (data not shown). The analysis of lipoprotein number, size and function identifies hepatic LXRα as an important regulator of lipoprotein metabolism and suggests that alterations in LDL and/or HDL function may contribute to the increased atherosclerosis observed in *Ldlr*$^{-/-}$/LivKO mice.

RCT is impaired in LivKO mice. The ability to excrete cholesterol from the liver into the bile is a critical step in the RCT pathway. Our studies in normal lipidemic mice indicate that the ability of LXR agonists to stimulate cholesterol excretion is lost in the absence of hepatic LXRα activity (data not shown); nevertheless T0901317 still retains anti-atherogenic activity (Figures 3.3 and 3.6). To examine RCT under hyperlipidemic conditions, mouse J774 cells loaded with ^{3}H-cholesterol and acetylated LDL *in vitro*

were injected into the peritoneal cavity of *Ldlr*$^{-/-}$/floxed and *Ldlr*$^{-/-}$/LivKO mice that been on Western diet for 9 weeks in the absence or presence of T0901317. The amount of ^{3}H in the plasma, liver and feces was determined 48 hours later (Figures 3.9 and 3.10). The ability of T0901317 to increase the fecal excretion of macrophage derived sterols was largely impaired in *Ldlr*$^{-/-}$/LivKO mice (Figure 3.8A, and 3.10A-C). Concurrently there is an increase in ^{3}H-sterol in the livers of *Ldlr*$^{-/-}$/LivKO mice (Figure 3.9B) indicating that hepatic LXRα is needed for agonist-dependent fecal excretion of macrophage-derived cholesterol. The ability of LXR agonists to increase the appearance of macrophage-derived ^{3}H-cholesterol in the plasma is thought to result from agonists acting on macrophage LXRs to enhance ABCA1 and ABCG1 dependent cholesterol efflux [561, 693]. Consistent with other studies [561, 693, 694], treatment of *Ldlr*$^{-/-}$/floxed mice with T0901317 produced a time-dependent increase in the level of ^{3}H-cholesterol in the plasma (Figure 3.9C). Interestingly, the level of ^{3}H-cholesterol in the plasma of *Ldlr*$^{-/-}$/LivKO mice was decreased relative to vehicle treated *Ldlr*$^{-/-}$/floxed controls and treatment with T0901317 had no effect (Figure 3.9C). For all 4 groups FPLC analysis indicated that the distribution of ^{3}H-tracer in the plasma exactly coincided with the distribution of bulk, unlabeled cholesterol (Figure 3.11). To determine if the decrease in plasma ^{3}H-cholesterol levels observed in *Ldlr*$^{-/-}$/LivKO animals resulted from impaired LXR transcriptional activity in macrophages, we recovered the J774 cells from the peritoneal cavity 48 hours after injection and quantitated ABCA1 mRNA levels in RNA isolated from these cells. As shown in Figure 3.9D, agonist treatment produced a similar increase in ABCA1 mRNA in cells recovered from either *Ldlr*$^{-/-}$/floxed or *Ldlr*$^{-/-}$/LivKO animals. Additionally, a similar induction of ABCA1 mRNA was observed in RNA isolated from whole blood

taken from animals on Western diet in the absence or presence of T0901317 for 10 weeks (Figure 3.10D). The gene expression analysis suggests that the inability of LXR agonist to increase the appearance of macrophage-derived cholesterol in the plasma of *Ldlr*$^{-/-}$/LivKO mice does not arise from a defect in macrophage LXR activity.

3.4 Discussion

LXRs function throughout the body to control cholesterol transport, catabolism and excretion [677]. This report describes the first conditional LXR knockout mouse constructed by selectively eliminating the LXRα subtype in hepatocytes (LivKO mice). When challenged with a 2% cholesterol diet, LivKO mice accumulated increased amounts of cholesterol in liver resulting from failure to induce hepatic cholesterol excretion and catabolism, highlighting the importance of liver LXRα activity to whole body cholesterol homeostasis. Similarly, the ability of synthetic LXR agonists to stimulate biliary cholesterol excretion, inhibit fractional cholesterol absorption and increase the output of neutral sterols in the feces was largely compromised in LivKO mice. Several recent studies have described a *trans*-intestinal pathway for cholesterol excretion that bypasses biliary excretion but nevertheless can be stimulated by LXR activation [577, 685, 686]. Our studies suggest that such a biliary-independent pathway makes only a minor contribution to LXR agonist-dependent cholesterol excretion.

Early studies with synthetic LXR agonists described increases in plasma triglycerides and plasma HDL cholesterol as two pharmacological responses to LXR activation [524, 583]. Analysis of LivKO mice indicates that these responses originate from unique sites. The LXR agonist-dependent increases in triglycerides were of hepatic origin and resulted from regulation of the genes encoding SREBP-1c and other enzymes involved in fatty acid and triglyceride synthesis. In contrast, hepatic deletion of LXRα had little effect on the ability of LXR agonists to elevate HDL cholesterol. Both the liver and intestine have been shown to contribute to the production of HDL [315, 579, 580, 689] and while the LXR agonist-dependent induction of *Abca1*, a protein required for HDL

biogenesis, was impaired in liver, induction of *Abca1* in the intestine was unchanged. The tissue-specific expression of *Abca1* observed in LivKO mice suggests that LXR activation in the intestine is sufficient to regulate HDL cholesterol levels. Consistent with our conclusion that an intestinal LXR activity is primarily responsible for elevating HDL cholesterol, previous studies indicate that expression of ABCA1 in the intestine is required for LXR agonist-dependent cholesterol increases [579] and that transgenic over-expression of a constitutively active LXRα (VP16-LXRα) in the intestine increases HDL [662]. HDL cholesterol levels inversely correlate with cardiovascular disease risk and the ability of LXR agonists to increase HDL cholesterol initially stimulated great interest in the therapeutic potential of such compounds [677]. The concurrent increase in lipogenesis, however, has dampened the enthusiasm for LXR agonists and slowed the progression of molecules into the clinic. Analysis of LivKO mice demonstrates that the lipogenic and HDL pathways are tissue specific and suggests that LXR ligands that specifically target the intestine, for instance by limiting systemic absorption or by rapid first-pass clearance, could have therapeutic value.

In mouse models of cardiovascular disease, treatment with LXR agonists decreases atherosclerosis. However, in these hyperlipidemic models LXR agonists have little or no effect on HDL cholesterol levels and this has led to the conclusion that the anti-atherogenic activity originates from increased macrophage cholesterol efflux and/or limiting inflammation in immune cells in atherosclerotic plaque [548, 549, 552, 628]. Indeed, selective deletion of LXRα in hematopoietic cells increased atherosclerosis in the *Ldlr*$^{-/-}$ background although the increase was not as great as that measured in *Ldlr*$^{-/-}$/*Lxra*$^{-/-}$ global knockout mice [549, 569, 628]. We now demonstrate that atherosclerosis was

substantially increased when LXRα was selectively eliminated in hepatocytes, identifying the liver as a critical site of LXRα-dependent anti-atherogenic activity. Our studies suggest that hepatic LXRα modulates lipoprotein particle number, size, and function in a manner that influences atherogenicity. In particular the ability of HDL to accept cholesterol from macrophages is defective in *Ldlr*$^{-/-}$/LivKO mice. These observations suggest that pharmacological strategies utilizing small molecules that inhibit hepatic LXRα activity to reduce lipogenesis may actually increase cardiovascular disease and should be explored with caution. Future studies that explore the effect of hepatic LXR activity on lipoprotein function in the presence of the cholesterol ester transfer protein (CETP), a lipoprotein particle remodeling enzyme expressed in humans but not mice [666], will be useful in this regard.

Despite the increased atherosclerosis observed in *Ldlr*$^{-/-}$/LivKO mice, treatment with T0901317 was still an effective preventive therapy indicating that extra-hepatic LXR activity can also be anti-atherogenic. Our *in vivo* RCT analysis further suggests that the ability of LXR agonists to stimulate the RCT pathway is significantly compromised in the absence of hepatic LXRα and is thus not necessary for the athero-preventive activity of LXR agonists. The efficacy of agonist treatment in LivKO mice therefore raises questions regarding the potential mechanisms and sites of action for the pharmacological activity of LXR agonists. In contrast to the liver, using bone marrow transplantations we have previously shown that LXR activity in hematopoietic cells is necessary for the anti-atherogenic activity of T0901317 [549]. A number of additional functions for LXRs in immune cells including the control of inflammation [640], endoplasmic reticulum stress [695], macrophage egress [696] and monocyte proliferation [697, 698]

could underlie the anti-atherogenic activity of LXR ligands. Finally, recent studies indicate that intestinal specific activation of LXRs using pharmacological or transgenic approaches can increase RCT and may beneficially impact atherosclerosis[648, 662]. The failure of LXR agonist treatment to increase the appearance of macrophage-derived cholesterol in the plasma of *Ldlr*$^{-/-}$/LivKO mice during the *in vivo* RCT assay further raises the possibility that impaired LXR activity in the liver can negatively affect macrophage cholesterol efflux in the periphery. The appearance of macrophage-derived ^{3}H-cholesterol in the plasma during the *in vivo* RCT assay, however, may not simply reflect the rate the macrophage cholesterol efflux. The re-entry of ^{3}H-cholesterol into the plasma compartment after up-take by the liver and/or intestine may also contribute to this measurement. Therefore we cannot rule out the possibility that LXR agonists do in fact promote macrophage cholesterol efflux in *Ldlr*$^{-/-}$/LivKO mice and that this activity is anti-atherogenic even when hepatic cholesterol excretion to the bile is inhibited. In summary, our characterization of LivKO mice demonstrates that while endogenous hepatic LXRα activity is essential for maintaining normal lipid and sterol homeostasis, pharmacologic strategies that bypass LXR activation in liver may still be of therapeutic benefit.

Table 3.1. Lipoprotein Particle Size and Number.

`	***Ldlr*$^{-/-}$/Floxed**	***Ldlr*$^{-/-}$/LivKO**
VLDL particles, total nmol/L	727 ± 128	341 ± 130*
Large VLDL (> 60 nm), nmol/L	15.7 ± 4.5	4.4 ± 1.3*
Medium VLDL (35-60 nm), nmol/L	179 ± 103	117 ± 81
Small VLDL (27-35 nm), nmol/L	533 ± 67	220 ± 98*
LDL particles, total nmol/L	1238 ± 257	1440 ± 463
IDL (23-27 nm), nmol/L	1181 ± 246	685 ± 212*
Large LDL (21.2-23 nm), nmol/L	0	0
Small LDL (18-21.2 nm), nmol/L	0	742 ± 364*
HDL particles, total μmol/L	34.6 ± 9.6	24 ± 2.7*
Large HDL (8.8-13 nm), μmol/L	14.2 ± 6.4	16.7 ± 4.6
Medium HDL (8.2-8.2 nm), μmol/L	0	0
Small HDL (7.3-8.2 nm), μmol/L	18 ± 3.2	7.3 ± 4.7*
HDL Cholesterol mg/dL	50.6 ± 25.8	60.3 ± 17.2

*Statistically significant difference between floxed and LivKO mice (n = 6/group; $p \leq 0.05$).

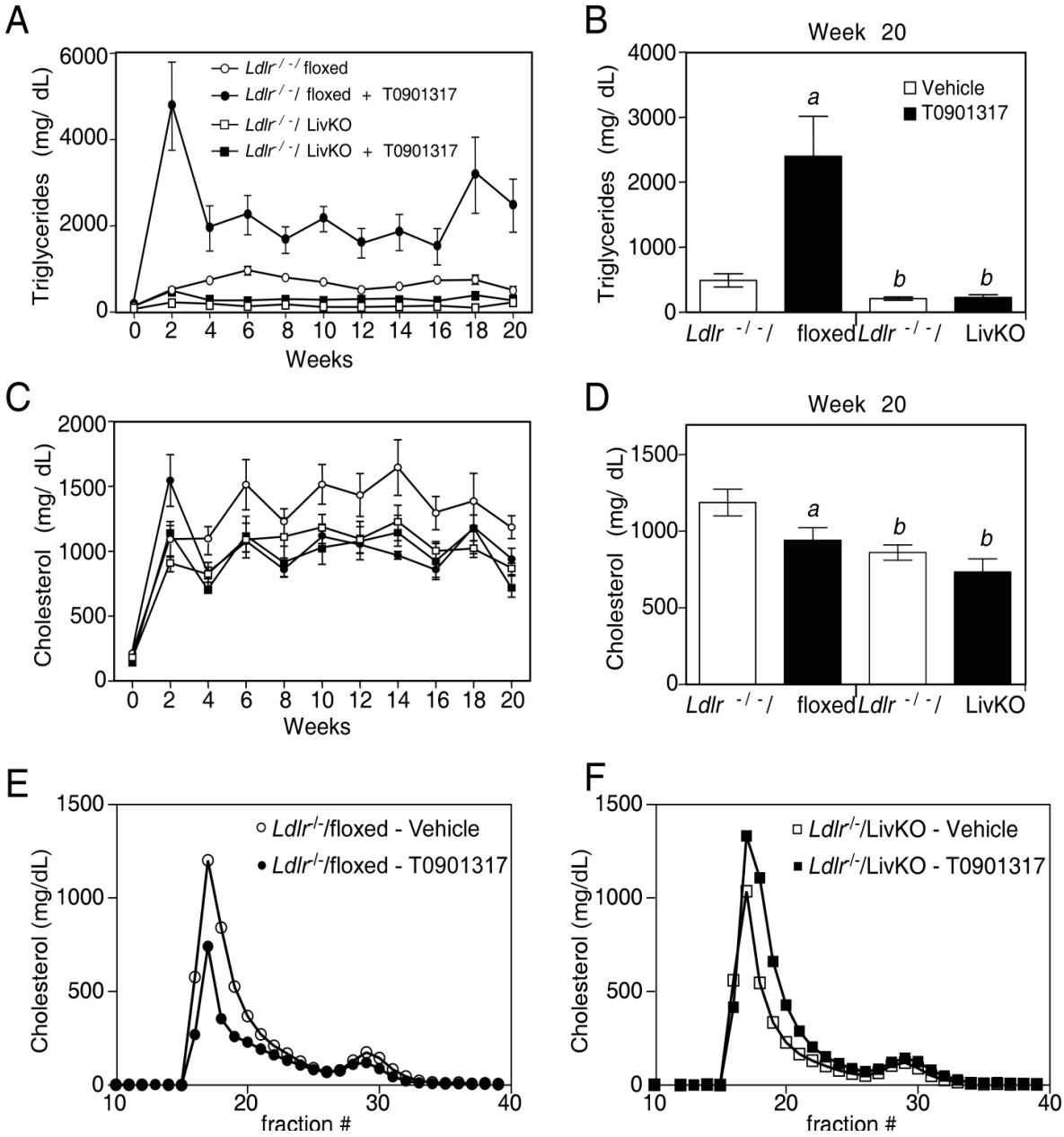

Figure 3.1 ***Plasma lipid levels in Ldlr$^{-/-}$/LivKO mice.***

Figure 3.1 ***Plasma lipid levels in Ldlr$^{-/-}$/LivKO mice.*** *Ldlr$^{-/-}$*/floxed and *Ldlr$^{-/-}$*/LivKO mice were fed a Western diet with or without 0.01% T0901317 for 20 weeks and **(A,B)** plasma triglycerides and **(C,D)** plasma total cholesterol levels were determined at 2 week intervals (n=5/group). Data are the mean ± SEM. d b, statistically significant difference between Flox and LivKO mice with the same treatment ($p \leq 0.05\%$). *Ldlr$^{-/-}$*/floxed **(E)** and *Ldlr$^{-/-}$*/LivKO **(F)** mice were fed a Western diet with or without 0.01% T0901317 for 10 weeks and FPLC analysis was carried out using pooled plasma (n=6/group) obtained from mice that had been fasted overnight. Elevated plasma triglycerides in samples from *Ldlr$^{-/-}$*/floxed mice treated with T0901317 resulted in a significant amount of non-HDL aggregating when samples were centrifuged to pellet particulate matter prior to loading the FPLC column; therefore, the non-HDL cholesterol levels measured by FPLC for *Ldlr$^{-/-}$*/floxed mice treated with T0901317 is likely an underestimate.

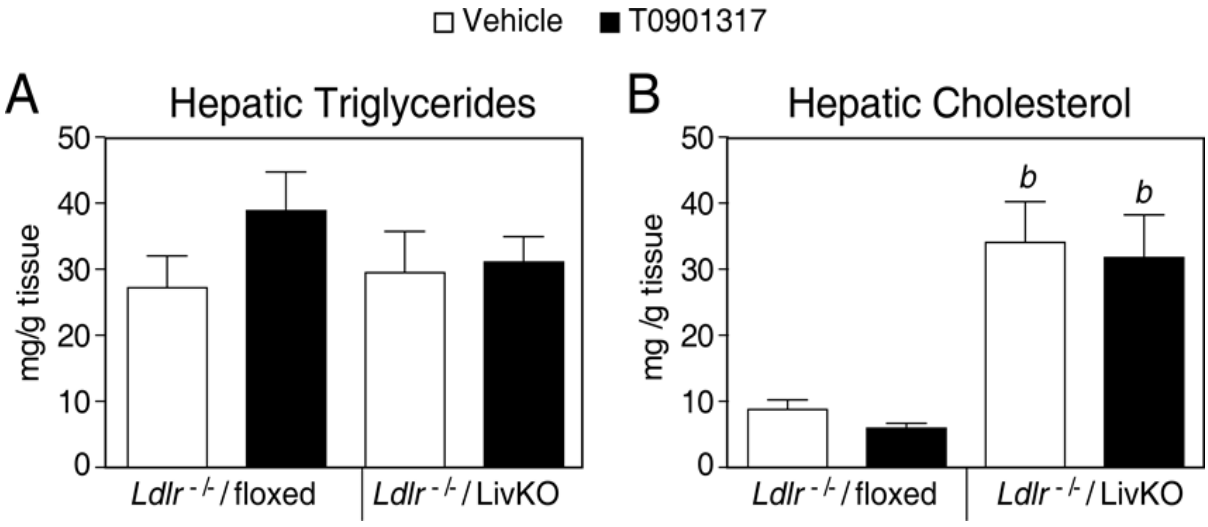

Figure 3.2 ***Hepatic lipid levels in Ldlr$^{-/-}$/LivKO mice.***

Figure 3.2 ***Hepatic lipid levels in Ldlr$^{-/-}$/LivKO mice.*** *Ldlr$^{-/-}$*/floxed and *Ldlr$^{-/-}$*/LivKO mice were fed a Western diet with or without 0.01% T0901317 for 20 weeks and hepatic **(A)** triglycerides and **(B)** cholesterol levels were determined at completion of the study (n=5/group; 3 male, 2 female). Data are the mean ± SEM. *b*, statistically significant difference between Flox and LivKO mice with the same treatment ($p \leq 0.05\%$).

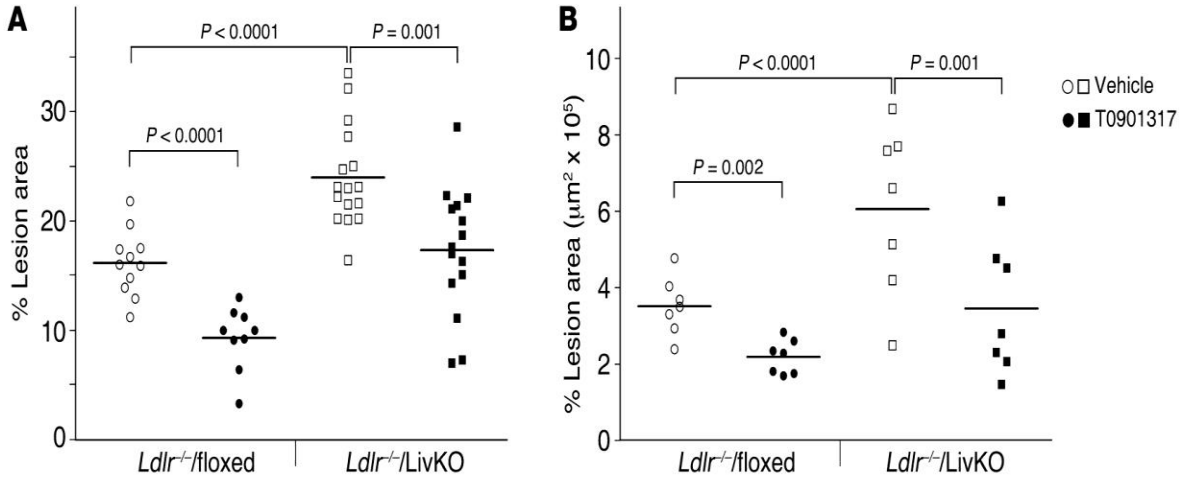

Figure 3.3 ***Atherosclerosis in Ldlr$^{-/-}$/LivKO mice.***

Figure 3.3 ***Atherosclerosis in Ldlr$^{-/-}$/LivKO mice.*** Mice were fed a Western diet with or without 0.01% T0901317 for 20 weeks, and atherosclerosis was quantitated. **(A)** En face analysis of the aorta was carried out as described in Methods. Ldlr$^{-/-}$/floxed (vehicle: n = 11, 6 male, 5 female; T0901317: n = 9, 5 male, 4 female). Ldlr$^{-/-}$/LivKO (vehicle: n = 16, 8 male, 8 female; T0901317: n = 15, 8 male, 7 female). **(B)** Lesion area was measured in serial sections of the aortic root (n = 7/group; 4 male, 3 female). Each data point represents an individual animal. The horizontal lines denote the mean of each group. There were no significant differences between sexes within any of the groups.

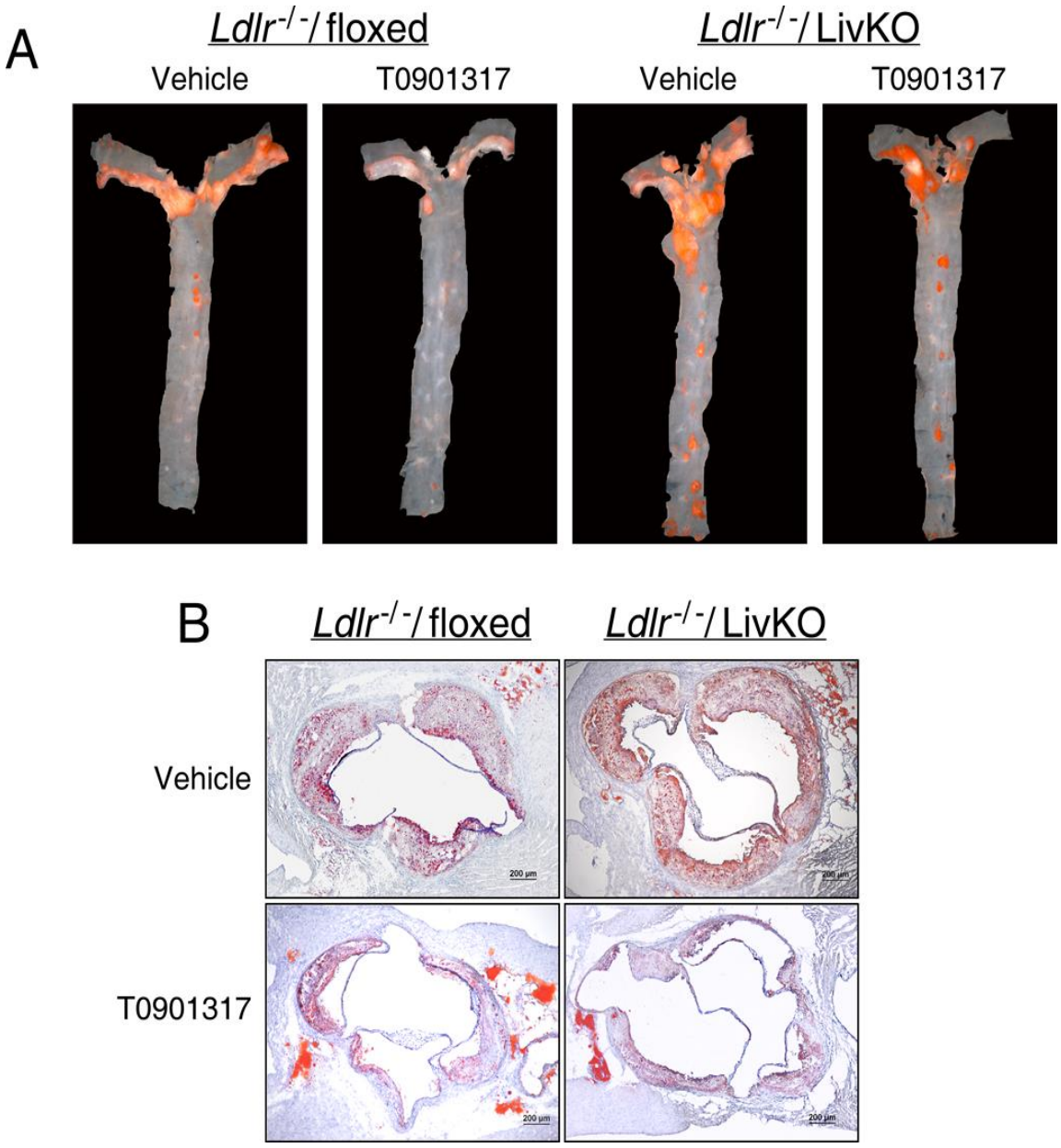

Figure 3.4 ***Representative en face and root section images.***

Figure 3.4 ***Representative en face and root section images.*** Representative Sudan IV stained aortas **(A)** and oil red O stained aortic root sections **(B)**.

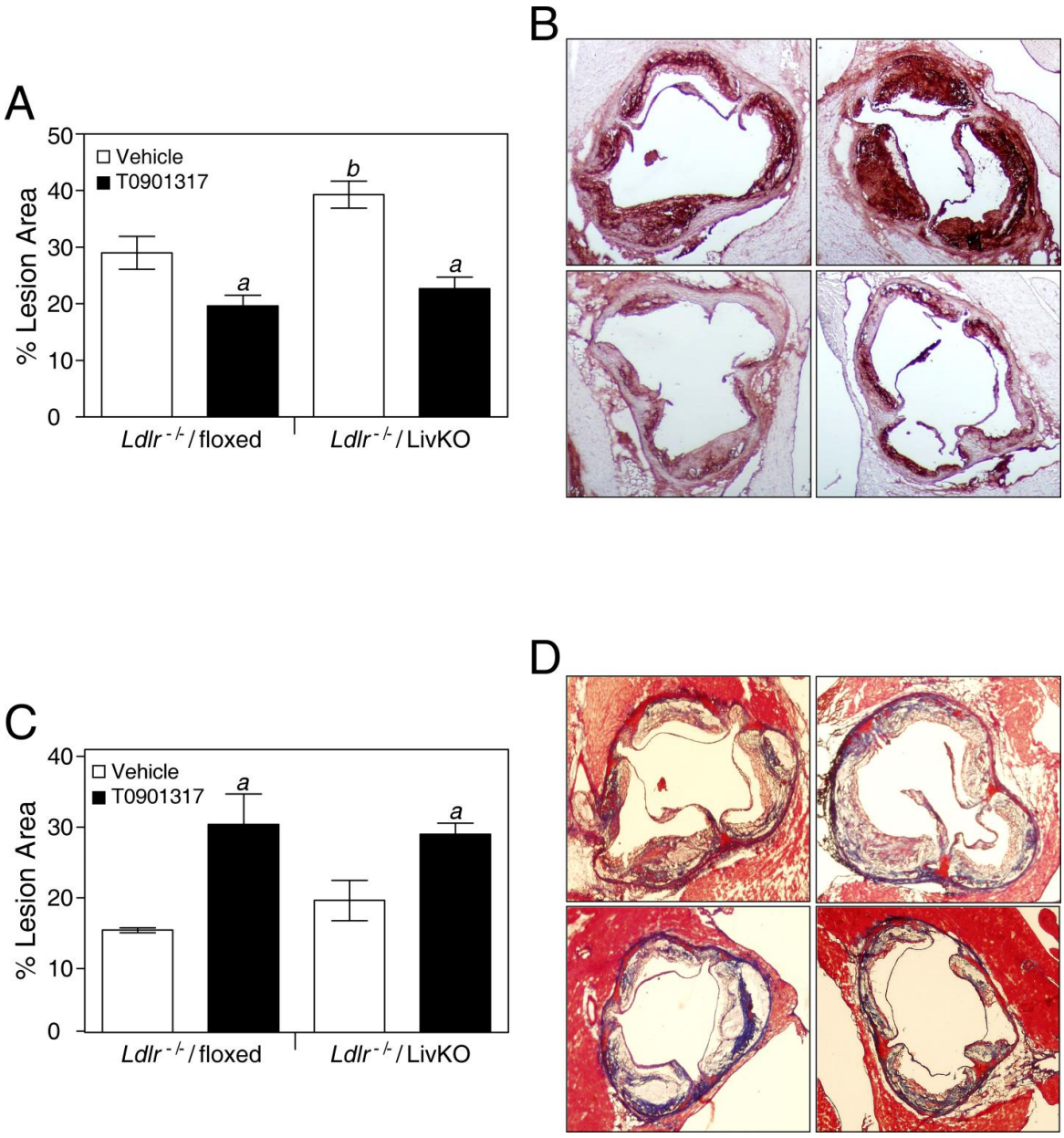

Figure 3.5 ***Macrophage and collagen staining.***

Figure 3.5 ***Macrophage and collagen staining.*** Aortic root sections from *Ldlr*$^{-/-}$/floxed and *Ldlr*$^{-/-}$/LivKO mice fed a Western diet with or without 0.01% T0901317 for 20 weeks were stained with antibodies to MOMA-2 to detect macrophages **(A,B)** or with trichrome to detect collagen **(C,D)**. Quantification was carried out as described in the Methods. Data are the mean ± SEM. *a*, statistically significant difference between vehicle and T0901317 treated animals of the same genotype ($p \leq 0.05\%$). *b*, statistically significant difference between Flox and LivKO mice with the same treatment ($p \leq 0.05\%$).

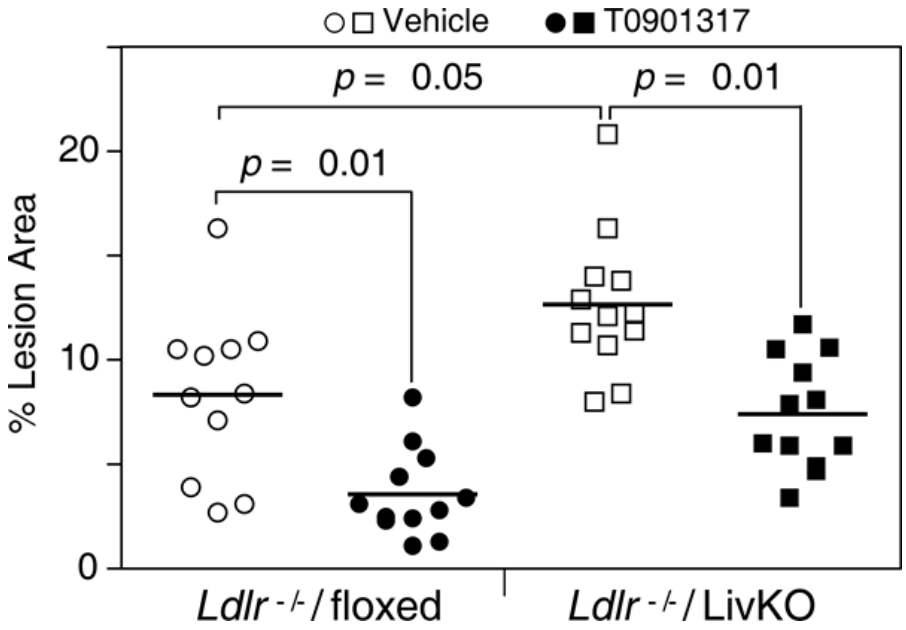

Figure 3.6 ***Atherosclerosis in Ldlr$^{-/-}$/LivKO mice on 10 weeks of Western diet.***

Figure 3.6 ***Atherosclerosis in Ldlr$^{-/-}$/LivKO mice on 10 weeks of Western diet.*** Mice were fed a Western diet with or without 0.01% T0901317 for 10 weeks and atherosclerosis was quantitated by en face analysis as described in the Methods. *Ldlr$^{-/-}$* /floxed (vehicle n=11; T0901317 n=12). *Ldlr$^{-/-}$*/LivKO (vehicle n=12; T0901317 n=12).

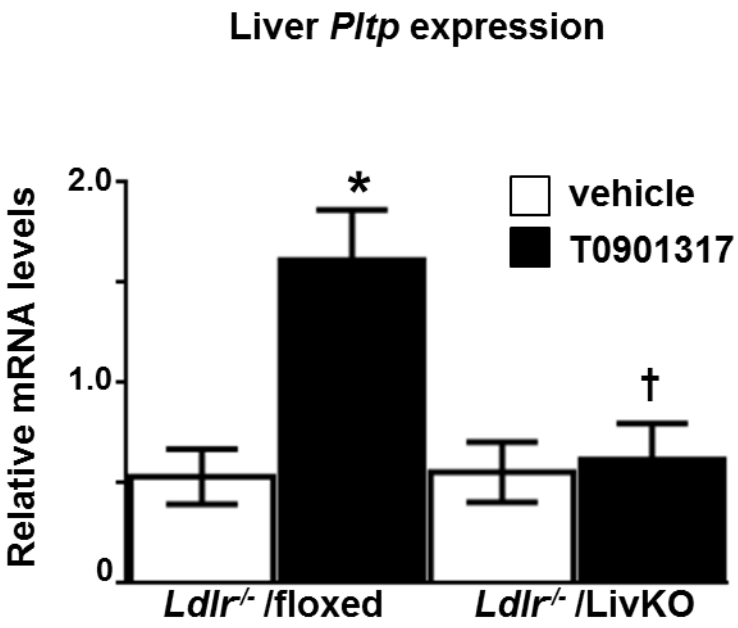

Figure 3.7 ***Pltp expression in Ldlr$^{-/-}$ /LivKO mice.***

Figure 3.7 ***Pltp expression in Ldlr$^{-/-}$ /LivKO mice.*** Total RNA was isolated from livers at the completion of *in vivo* RCT study and mRNA levels were measured by quantitative real-time PCR as described in Materials and Methods. Data is mean ± SEM, (n=5-6/group). *Statistically significant difference between vehicle and T0901317-treated animals of the same genotype ($p \leq 0.05\%$). †Statistically significant difference between Floxed and LivKO with the same treatment ($p \leq 0.05\%$).

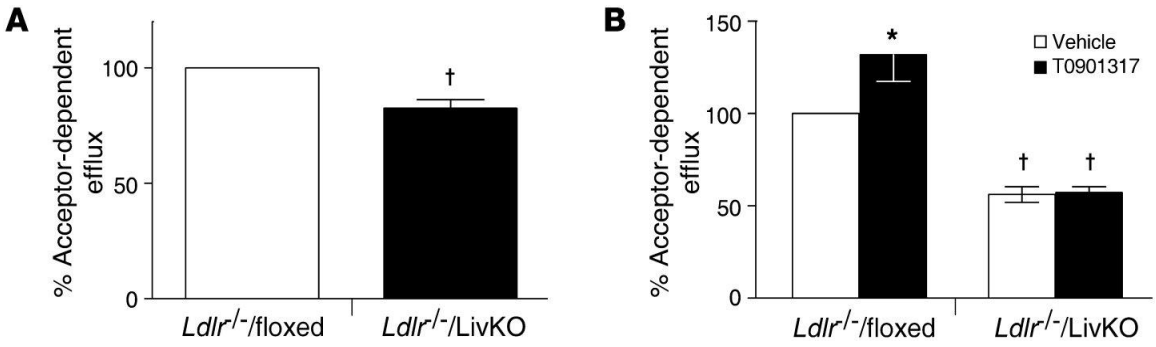

Figure 3.8 ***Cholesterol efflux in RAW264.7 cells.***

Figure 3.8 ***Cholesterol efflux in RAW264.7 cells.*** ^{3}H-Cholesterol–loaded RAW264.7 cells were cultured with **(A)** 0.03% plasma or **(B)** FPLC-purified HDL isolated from mice fed a Western diet with or without 0.01% T0901317 for 9 weeks, and cholesterol efflux was determined as described in Methods. Efflux to plasma or HDL isolated from Ldlr$^{-/-}$/floxed mice fed a Western diet without T0901317 was set as 100%. Data are the average of 2 independent experiments and expressed as mean ± SEM. Data from the experiments with FPLC-purified HDL were normalized to the amount of apoAI added. *$P \leq 0.05$ between vehicle- and T0901317-treated animals of the same genotype; $^{\dagger}P \leq 0.05$ between floxed and LivKO mice.

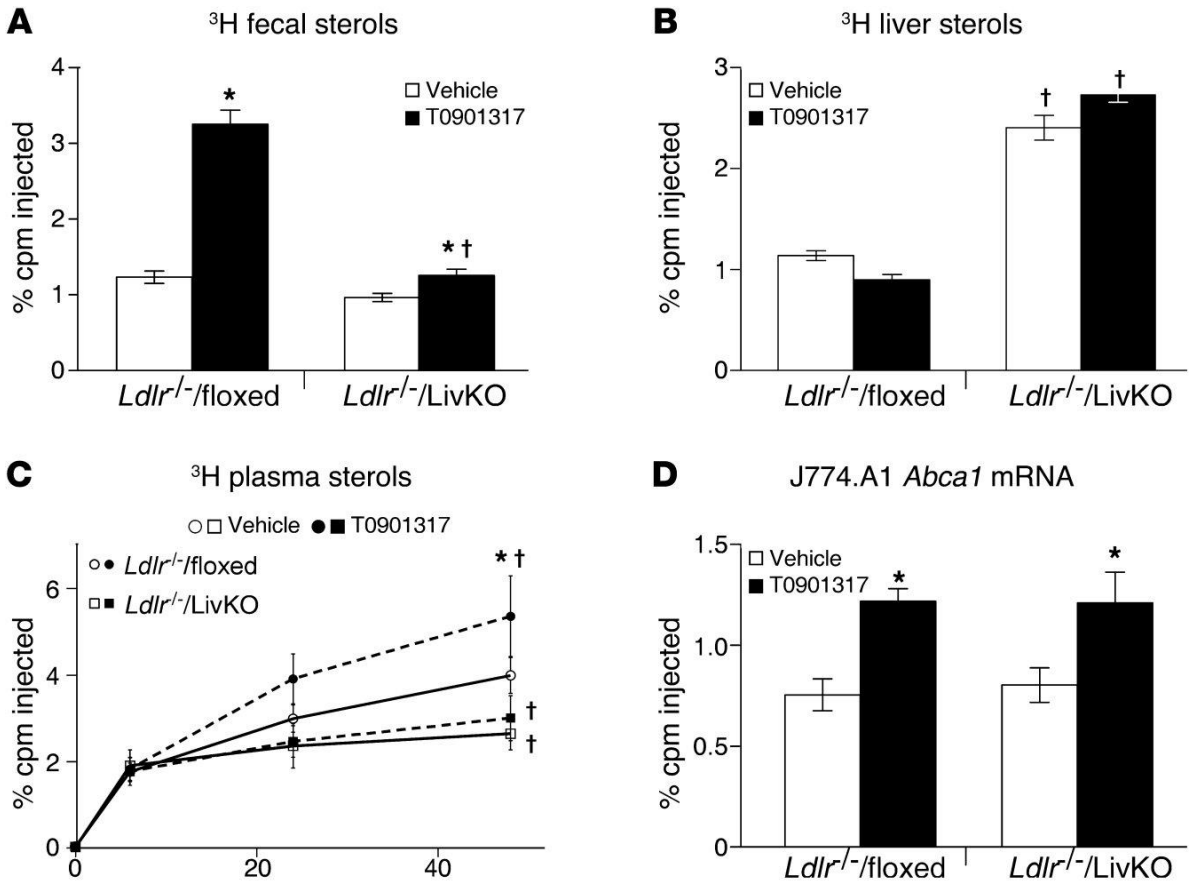

Figure 3.9 In vivo *RCT in Ldlr$^{-/-}$ /LivKO.*

Figure 3.9 In vivo *RCT in Ldlr$^{-/-}$ /LivKO*. ^{3}H-cholesterol and acetylated LDL–loaded J774 macrophages were injected into mice fed a Western diet with or without 0.01% T0901317 for 10 weeks (n = 6/group), and the amount of ^{3}H tracer in feces (**A**), liver (**B**), and plasma (**C**) was determined as described in Methods. (**D**) Total RNA was isolated from recovered J774 macrophages, and the mRNA levels of Abca1 were measured by quantitative real-time PCR. Data are mean ± SEM. *$P \leq 0.05$ between vehicle- and T0901317-treated animals of the same genotype; $^{\dagger}P \leq 0.05$ between floxed and LivKO mice with the same treatment.

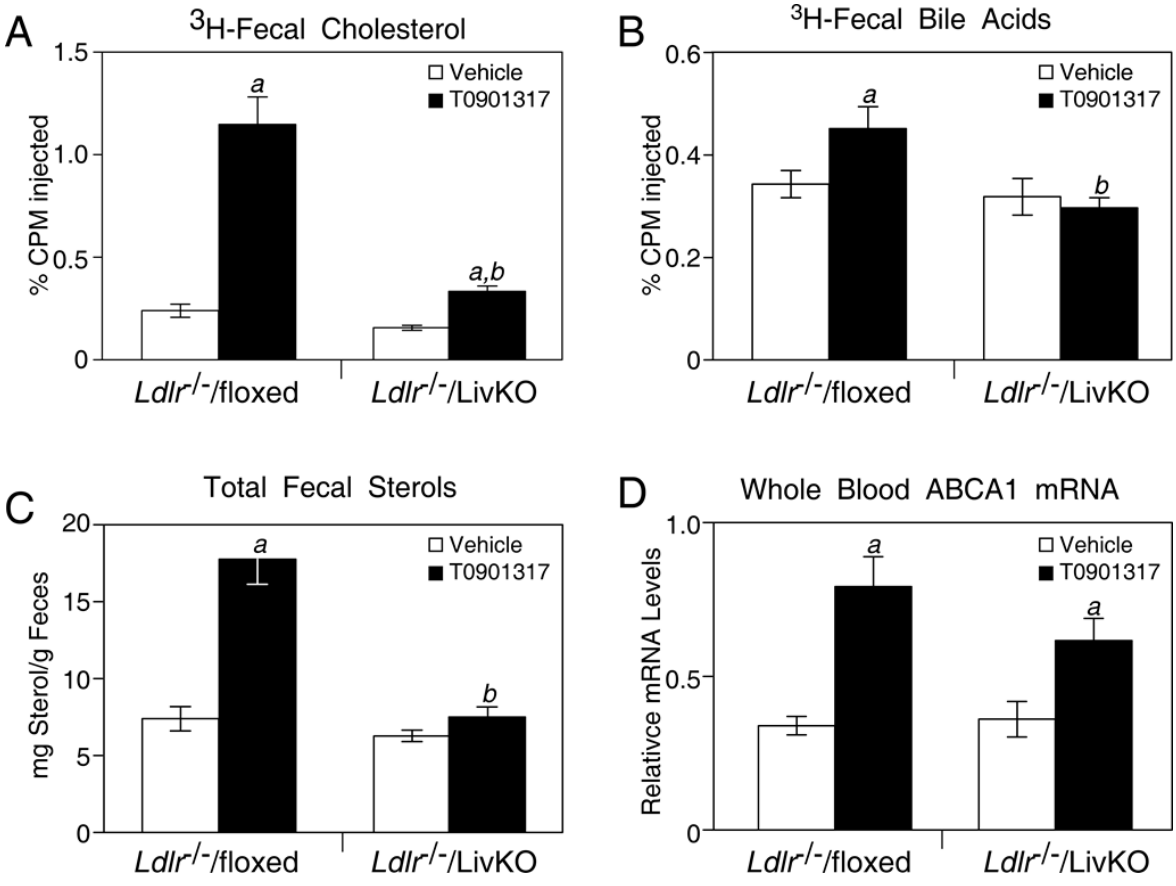

Figure 3.10 ***In vivo RCT, total fecal sterols and whole blood gene expression.***

Figure 3.10 ***In vivo RCT, total fecal sterols and whole blood gene expression.*** Mice were fed a Western diet with or without 0.01% T0901317 for 9 weeks. **(A,B)** *In vivo* RCT analysis was carried out as described in the Methods (n=6/group) and the levels of ^{3}H-cholesterol **(A)** and ^{3}H-bile acids **(B)** was determined. **(C)** Total fecal sterols were determined as described in the Methods from feces collected just prior to initiating the *in vivo* RCT experiment. **(D)** Total RNA was isolated from whole blood as described in the Methods and the mRNA levels of ABCA1 were measured by quantitative real-time PCR. Data are the mean ± SEM. *a*, statistically significant difference between vehicle and T0901317 treated animals of the same genotype ($p \leq 0.05\%$). *b*, statistically significant difference between Flox and LivKO mice with the same treatment ($p \leq 0.05\%$).

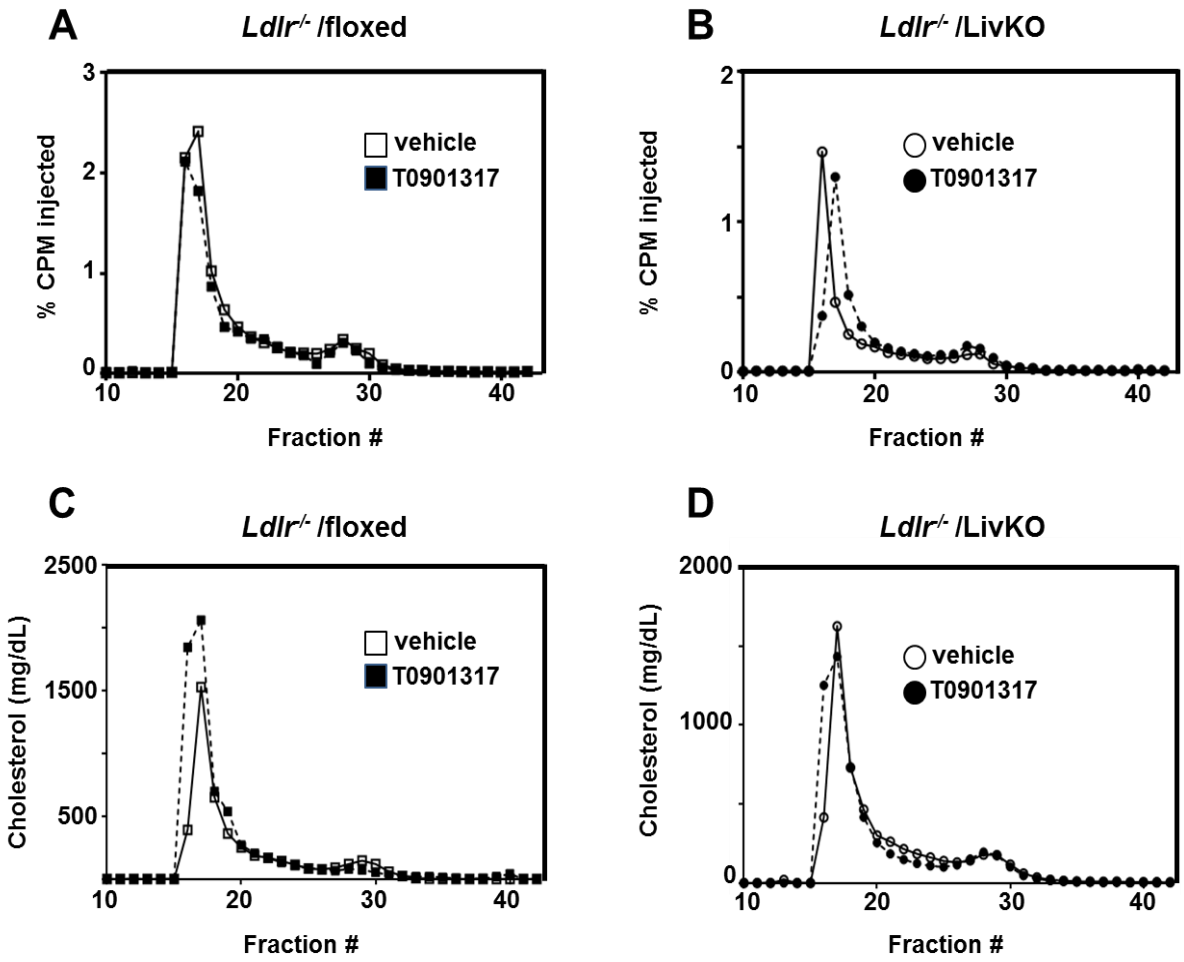

Figure 3.11 ***Radiolabeled cholesterol is redistributed among lipoprotein particles***

Figure 3.11 ***Radiolabeled cholesterol is redistributed among lipoprotein particles.*** At completion of the *in vivo* RCT study, pooled plasma samples were subjected to FPLC and the ^{3}H content (**A** and **B**) was determined (n=6/group). Following 10 weeks of Western diet with or without T0901317 (0.01%) plasma was pooled and subjected to FPLC and the cholesterol content of each fraction was measured as described in the Materials and Methods (**C** and **D**).

Chapter 4: Materials and Methods

4.1 Materials and Methods for Chapter 2

Reagents. LXR agonist T0901317 was purchased from Cayman Chemical. ^{3}H-cholesterol was purchased from Perkin Elmer. ^{14C}Cholic acid was purchased from American Radiolabeled Chemicals Inc.

Animal experiments. All animal experiments were approved by the Institutional Animal Care and Research Advisory Committee of the University of Virginia. Lxr$\alpha^{-/-}$/Lxr$\beta^{-/-}$ and Lxr$\alpha^{fl/fl}$/albumin-Cre mice have been described previously[529, 628]. Male CETP transgenic mice (The Jackson Laboratory) were bred with female C57Bl6/J (The Jackson Laboratory) to generate CETP transgenic (CETP^{+}) and CETP^{-} littermate controls. All animals were housed in a temperature-controlled environment with 12-hour light/12-hour dark cycles. Age-matched mice had free access to water and were fed standard rodent chow (TD 7001, Harlan Teklad) or a 0.2% cholesterol diet (TD 07798, Harlan Teklad). For 0.2% cholesterol diet experiments, animals were switched from standard chow to cholesterol diet at 8 weeks of age and experiments carried out following 4 weeks of diet. Animals were treated with vehicle (80% polyethylene glycol, 20% Tween-80) or LXR agonist T0901317 (10 mg per kg of body weight) by oral gavage once per day in the morning. Samples were drawn or experiments were initiated 3 hours after the 3rd dose (in vivo RCT studies) or 5th dose (plasma analysis and FPLC studies).

Plasma Analyses. Blood was collected into EDTA-coated tubes (Starstedt). Plasma was separated by centrifugation and assayed for total cholesterol (Thermo Scientific), triglycerides (Pointe Scientific), HDL-cholesterol (Thermo Scientific), CETP activity (Sigma Aldrich), PLTP activity (Roar Biomedical Inc.), aspartate aminotransferase

(AST) activity (Pointe Scientific) activity, alanine aminotransferase (ALT) activity (Thermo Scientific) and APOA1 levels (Novatein Biosciences). Plasma lipoprotein levels in pooled plasma samples (n=4-6/group) were analyzed by FPLC using a Superose G6 10/300 GL column (GE Healthcare), and assayed for total cholesterol per fraction by enzymatic analysis (Thermo Scientific). FPLC purified fractions containing peak HDL-cholesterol (n=3 fractions) were pooled for in vitro experiments. FPLC purified HDL was assayed for total phospholipid by calorimetric kit (Wako).

Western blotting. FPLC purified HDL samples (12.5 μl) in triplicate were resolved on 12% SDS-polyacrylamide gels and transferred to PVDF membranes (Millipore). Membranes were then hybridized with goat anti–mouse APOA1 antibody (Abcam 7614) at a 1:1000 dilution, followed by secondary antibody incubation at a 1:5,000 dilution. APOA1 protein was detected by chemiluminescence and quantitated with ImageQuant software.

Liver cholesterol and triglycerides. Liver samples (0.1 g) were homogenized in 2 ml Folch (chloroform/methanol, 2:1, v/v) with a polytron homogenizer. The organic phase was separated and then dried under nitrogen. Samples were reconstituted in isopropranol:Triton-X100 (9:1 v/v) and aliquots subjected to colorimetric enzymatic assays for total cholesterol (Thermo Scientific) or triglycerides (Pointe Scientific).

Quantitative real-time PCR analysis. Total RNA was extracted from ~100 mg pieces of tissue (liver and small intestine) using a microbead tissue homogenizer and PureZOL (Bio-Rad)/chloroform extraction. Total RNA was isolated from aqueous phase using an RNeasy kit (QIAGEN). RNA was treated with DNase I and reverse transcribed into cDNA with random hexamers using a High-Capacity cDNA Reverse Transcription kit

(Life Technologies). RT-qPCR reactions contained 25 ng of cDNA, 385 nM of each primer, and 6.25 µl of SYBR Green Supermix (Bio-Rad) and were carried out in triplicate using a Bio-Rad MyiQ instrument. Relative mRNA levels were calculated using the comparative Ct method and normalized to cyclophilin.

Cholesterol efflux experiments. RAW264.7 cells were plated in 96-well plates (2×10^5 cells/well) and 24 hours later labeled with 1 µCi/ml ^{3}H-cholesterol (PerkinElmer) in DMEM plus 1% FBS media for 18-24 hours. Radiolabeled cells were washed with pre-warmed PBS and incubated for 18-24 hours with serum-free media containing 0.03% pooled plasma (n=5-7 animals/group) or with 25% FPLC-purified HDL normalized to APOA1 levels as determined by Western blotting as cholesterol acceptors. For each individual efflux experiment using FPLC purified HDL, triplicate samples of all the HDL samples being compared were quantitated on the same western blot. Following incubation with acceptors, media was collected and radioactivity was measured by liquid scintillation counting. Cells were washed with PBS and lysed in 100 µl of 0.2 N NaOH, and radioactivity in cell lysates was quantitated by liquid scintillation counting. Cholesterol efflux was expressed as percentage of cpm in the medium divided by the total counts ($cpm_{media}/[cpm_{media} + cpm_{cell}]$). Acceptor-dependent efflux was determined by subtracting the efflux of vehicle cells cultured without acceptor. APOA1 (10 µg/ml) or HDL (15 µg/ml) was included as positive control.

In vivo RCT. In vivo RCT experiments were carried out as described by Naik et al[561]. Animals were on either chow or 0.2% cholesterol diet as indicated. Three days prior to and for the duration of the experiment mice were gavaged with vehicle or T0901317 (10 mpk). Bone marrow derived macrophages were loaded with 25 µg/ml acetylated LDL

and 5 μCi/ml ^{3}H-cholesterol for 48 hours in vitro. Cholesterol-loaded cells were injected into the peritoneal cavity of mice (~4.5 x 10^{6} cells/mouse, 3 x 10^{6} cpm, n=6/group), which were housed individually for the duration of the experiment. Blood was collected at 6, 24, and 48 hours after injection, and the ^{3}H-cholesterol in triplicate plasma samples (10 ul aliquots) was determined by scintillation counting. Frozen livers, gonadal fat pads, testes, and quadriceps skeletal mucsle were ground with mortar and pestle and lipids extracted from duplicate 100 mg samples by standard Folch extraction. Lipids were resuspended in 1ml of liquid scintillation fluid and the ^{3}H-cholesterol levels in triplicate 200 ul aliquots was determined by scintillation counting. Feces was collected at 48 hours, homogenized in 50% EtOH by polytron homogenizer and ^{3}H-sterol levels determined by scintillation counting in 200 ul aliquots in triplicate. To measure ^{3}H-cholesterol and ^{3}H-bile acid in feces, 2 mL of homogenized samples was combined with 2 mL ethanol, 0.03μCi of ^{14}C-cholic acid as an internal standard, and 400 μL NaOH. The samples were saponified at 95°C for 2 hours, cooled to room temperature and cholesterol separated from bile acids by extracting 2 times with 6 mL hexane. The extracts were pooled, evaporated, resuspended in toluene and ^{3}H-cholesterol levels were determined by scintillation counting. To extract bile acids, the remaining aqueous fraction after the hexane extractions was acidified with concentrated HCl and then extracted 2 times with 6 mL ethyl acetate. The extracts were pooled together, evaporated, resuspended in ethyl acetate, and and ^{3}H-bile acids levels were determined by scintillation counting and normalized to the recovery of ^{14}C-cholic acid.

To measure gene expression in recovered BMMs, 48 hours after ^{3}H-macrophage injection the peritoneal cavity of the mouse was flushed with PBS and cells were

collected. Recovered cells were lysed with PureZOL (Bio-Rad), extracted with chloroform, and total RNA was isolated from the aqueous layer using an RNeasy kit (QIAGEN). Quantitative RT-PCR was carried out as described above.

Statistics. Results were analyzed by 1-way ANOVA with Tukey's post-test using GraphPad Prism (GraphPad Software); p values of 0.05 or less were considered significant.

4.2 Material and Methods for Chapter 3

Reagents. LXR agonist T0901317 was purchased from Cayman Chemical (Ann Arbor, Michigan).

Animal experiments. All animal experiments were approved by the Institutional Animal Care and Research Advisory Committee at the University of Virginia. Floxed LXRα mice were bred with C57BL/6J Albumin-Cre transgenic mice (Jackson Laboratories) to generate liver-specific knock out animals (LivKO). The littermates carrying flox/flox allele were used as their control (Flox).

Ldlr$^{-/-}$/*Lxrα*$^{fl/fl}$/Albumin-CRE^{-} and *Ldlr*$^{-/-}$/*Lxrα*$^{fl/fl}$/Albumin-CRE^{+} mice were created by mating *Lxrα*$^{fl/fl}$/Albumin-CRE^{+} mice with *Ldlr*$^{-/-}$ mice (male B6.129S7-*Ldlr*^{tm1Her}/J) purchased from Jackson Laboratories. Mice were fed standard chow ad libitum until put on study. To examine atherosclerosis in *Ldlr*$^{-/-}$/*Lxrα*$^{fl/fl}$/Albumin-CRE^{-} and *Ldlr*$^{-/-}$/*Lxrα*$^{fl/fl}$/Albumin-CRE^{+} mice, 8-9 week old animals were placed on a Western diet (21% fat wt/wt, 0.15% cholesterol wt/wt; Test Diet 57BD) with or with 0.01% T0901317 added to the diet for 10 or 20 weeks.

Plasma analyses. Blood was collected into EDTA-coated tubes (Starstedt). Plasma was separated by centrifugation and assayed for total cholesterol (Thermo Scientific), triglycerides (Thermo Scientific). Plasma lipoprotein levels were analyzed by fast protein liquid chromatography using Superose HR6 columns followed by enzymatic assays for total cholesterol and triglycerides.

Liver cholesterol and triglycerides. Liver lipids were extracted in Folch (chloroform: methanol, 2:1, v/v) [572]. Liver samples (0.1 g) were homogenized in 4 ml of Folch. Extracts were then washed once with 1 ml of 50 mM NaCl and twice with 1 ml of 0.36M $CaCl_2$/methanol. The organic phase was separated and brought up to 5 ml with chloroform. Fifty microliters of each sample and ten microliters of standards were mixed with 10 mL of 50% Triton X-114 in chloroform (v/v). Samples were air dried and then subjected to colorimetric enzymatic assays for total cholesterol (Thermo Scientific) or triglycerides (Thermo Scientific).

Quantitative real-time PCR analysis. Total RNA was extracted from liver and small intestine using RNA STAT-60 (Tel-Test, Inc.). RNA was treated with DNase I and then reverse transcribed into cDNA with random hexamers using the SuperScript II First-Strand Synthesis System (Invitrogen). Primers for each gene were designed using Primer Express Software (Applied Biosystems) and were validated as previously described [699]. RT-qPCR reactions contained 25 ng of cDNA, 150 nM of each primer, and 5 ml of SYBR GreenER PCR Master Mix (Invitrogen) and were carried out in triplicate using an Applied Biosystems Prism 7900HT instrument. Relative mRNA levels were calculated using the comparative C_T method normalized to cyclophilin.

Quantitation of Atherosclerosis. Atherosclerosis, macrophage levels and collagen staining in root sections and *en face* preparations were quantitated as previously described [549, 569, 700].

NMR lipoprotein measurements. *Ldlr*$^{-/-}$/*Lxr*$\alpha^{fl/fl}$/Albumin-CRE^{-} and *Ldlr*$^{-/-}$ /*Lxr*$\alpha^{fl/fl}$/Albumin-CRE^{+} mice were fed a Western diet with or without 0.01% T0901317 as described above (n=6/group). After 10 weeks, mice were euthanized, blood was collected into EDTA coated tubes and centrifuged at 3000 rpm for 15 minutes at 4°C to prepare plasma. Lipoprotein particle concentrations and size in plasma samples were analyzed by nuclear magnetic resonance (NMR) spectroscopy by LipoScience Inc. (Raleigh, NC) [690].

Cholesterol efflux experiments. RAW264.7 cells were plated in 96 well plates ($2x10^5$ cells/well) and 24 hours later were labeled with 1μCi/ml ^{3}H-Cholesterol (Perkin Elmer) in DMEM + 1% FBS media for 18-24 hours. Radiolabeled cells were washed with pre-warmed PBS and incubated for 6 hours with serum-free media containing 0.03% pooled plasma (n=6 animals/group) as cholesterol acceptors. Following incubation with acceptors, media was collected and radioactivity was measured by liquid scintillation counting. Cells were washed with PBS, lysed in 100 ul of 0.2 N NaOH and radioactivity in cell lysates was quantitated by liquid scintillation counting. Cholesterol efflux was expressed as percentage of counts/minute (CPM) in the medium divided by the total counts (CPM_{media} / (CPM_{media} + CPM_{cell}). Acceptor dependent efflux was determined by subtracting the efflux of vehicle cells cultured without acceptor. Apolipoprotein AI (10 μg/ml) or HDL (15μg/ml) was included as positive controls. Data is expressed as mean ± SEM of at least 8 wells/treatment.

Macrophage RCT experiments. *In* vivo RCT experiments were carried out as described by Naik et al.[561] in *Ldlr*$^{-/-}$/*Lxr*$\alpha^{fl/fl}$/Albumin-CRE^{-} and *Ldlr*$^{-/-}$/*Lxr*$\alpha^{fl/fl}$/Albumin-CRE^{+} mice fed a Western diet with or without 0.01% T0901317 for 9 weeks. Briefly, J774.A1 cells were loaded with 25 μg/ml acetylated low-density lipoprotein and 5 μCi/ mL ^{3}H-cholesterol for 48 hours *in vitro*. Cholesterol loaded cells were injected into the peritoneal cavity of mice (4.5 x 10^{6} cells/mouse, 3 x 10^{6} CPM, n = 6/group) which were housed individually for the duration of the experiment. Blood was collected at 6, 24, and 48 hours after injection and the ^{3}H-choelsterol in triplicate plasma samples was determined by scintillation counting. Levels of ^{3}H tracer in the liver, ^{3}H-total fecal sterols, fecal ^{3}H-choelsterol and fecal ^{3}H-bile acids was measured as described by Naik et al. [561].

To measure gene expression in J774.A1 cells recovered from the peritoneal cavity of mice, cells were collected from the peritoneal cavity 48 hours after injection, lysed with Purzol (Biorad), extracted with chloroform and total RNA was isolated from the aqueous layer using an RNeasy kit (Qiagen). RNA was treated with DNase I and then reverse transcribed into cDNA with random hexamers using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems). RT-qPCR reactions contained 20 ng of cDNA, 385 nM of each primer, and 8 μl of iQ SYBR Green Supermix (Biorad) and were carried out in triplicate using a Biorad MyiQ instrument. Relative mRNA levels were calculated using the comparative C_T method normalized to cyclophilin.

Gene expression in whole blood and liver. Fresh whole blood (approximately 350 μl) was collected in heparinized capillary tubes, transferred to ependorf tubes and cells were lysed with 0.7 ml Purazol (Biorad). Following lysis, 150 ul of chloroform was added and total RNA was isolated from the aqueous layer using an RNeasy kit (Qiagen). ABCA1

mRNA levels were measured and normalized to cyclophilin as described above. Liver samples were lysed in Purazol (Biorad), extracted with chloroform and total RNA was isolated from the aqueous layer using an RNeasy kit (Qiagen). PLTP mRNA were measured and normalized to cyclophilin as described above.

Statistical analyses. Results were analyzed by one-way analysis of variance (ANOVA) or Student's unpaired t-test, using GraphPad Prism (GraphPad Software, Inc.).

Table 4.1 Oligonucleotides for real-time PCR.

Mouse cyclophilin	5’ CGATGACGAGCCCTTGG 3’ 5’ TCTGCTGTCTTTGGAACTTTGTC 3’
Mouse ABCA1	5’ GCTCTCAGGTGGGATGCAG 3’ 5’ GGCTCGTCCAGAATGACAAC 3’
Mouse ABCG1	5’ ATCTGAGGGATCTGGGTCTGA 3’ 5’ CCTGATGCCACTTCCATGA 3’
Human CETP	5’ GGCCAATCAAGTATGGGTTG 3’ 5’ ACAGACACGTTCTGAATGGAGA 3’
Mouse ABCG8	5’ TGCCCACCTTCCACATGTC 3’ 5’ ATGAAGCCGGCAGTAAGGTAGA 3’
Mouse ABCG5	5’ ATGAAGCCGGCAGTAAGGTAGA 3’ 5’ GGCAGGTTTTCTCGATGAACTG 3’
Mouse ATF4	5’ GCAGTGTTGCTGTAACGGACA 3’ 5’ CGCTGTTCAGGAAGCTCATCT 3’
mXBP1 (s)	5’ GAGTCCGCAGCAGGTG 3’ 5’ GTGTCAGAGTCCATGGGA 3’
Mouse CHOP	5’ CCACCACACCTGAAAGCAGAA 3’ 5’ AGGTGAAAGGCAGGGACTCA 3’
Mouse BIP	5’ TTCAGCCAATTATCAGCAAACTCT 3’ 5’ TTTTCTGATGTATCCTCTTCACCAGT 3’
Mouse TNFα	5’ CTGAGGTCAATCTGCCCAAGTAC 3’ 5’ CTTCACAGAGCAATGACTCCAAAG 3’
Mouse IL-1β	5’ GGAGAACCAAGCAACGACAAAATA 3’ 5’ TGGGGAACTCTGCAGACTCAAAC 3’
Mouse CD36	5’ ATGGGCTGTGATCGGAACTG 3’ 5’ TTTGCCACGTCATCTGGGTTT 3’
Mouse LSR	5’ CTACAACCCCTATGTGGAGTGC 3’ 5’ CTGCCCTGGTAGTAGTCTCCC 3’
Mouse LRP1	5’ CCACTATGGATGCCCCTAAAAC 3’ 5’ GCAATCTCTTTCACCGTCACA 3’
Mouse SRB1	5’ TTTGGAGTGGTAGTAAAAAGGGC 3’ 5’ TGACATCAGGGACTCAGAGTAG 3’
Mouse LDLR	5’ TCAGACGAACAAGGCTGTCC 3’ 5’ CCATCTAGGCAATCTCGGTCTC 3’
Mouse PLTP	5’ TGGGACGGTGTTGCTCAA 3’ 5’ TCGATGCCCACGAGATCA 3’

Chapter 5: CONCLUSION AND FUTURE DIRECTIONS

Cardiovascular disease is a leading cause of death worldwide and the morbidity and mortality from this disease is only likely to increase as rest of the world westernizes. Statins, likely the most widely prescribed drug ever developed, are the current standard of care for the treatment of cardiovascular disease. While statins are quite effective for reducing plasma LDL-C levels and subsequently lowering cardiovascular risk many patients with cardiovascular disease who are on statin therapy still go on to develop major adverse cardiac events (stroke, myocardial infarction, etc.). This high residual risk among patients signifies the importance of uncovering other complementary or alternative approaches for the treatment of cardiovascular disease.

The liver X receptors (LXRs), are key modulators of cholesterol homeostasis throughout the body and pharmacological activation of LXRs has been shown to reduce atherosclerosis in several animal models. Much effort has been undertaken to uncover the potential anti-atherogenic function of LXRs in hopes of developing novel therapeutic targets for CVD that could either complement or supplant statins. The discovery that LXRs control macrophage cholesterol efflux via direct regulation of ABCA1, ABCG1, and APOE[570, 640] suggested a simple hypothesis for the cardio-protective effect of LXR activation based on promoting cholesterol efflux from macrophage foam cells to HDL; the first step of the RCT pathway. This hypothesis is supported by the finding that macrophage LXR activity is required for the anti-atherogenic activity of LXR agonists[549]. However, LXRs also modulate other key steps in the RCT pathway, namely increasing HDL-C (intestine), promoting cholesterol secretion (liver), and blocking cholesterol absorption (intestine).

The propensity of LXR agonists to increase plasma triglyceride levels through activation of LXRα in the liver presented a major challenge however for researchers interested in developing LXR-targeted therapeutics. There has been great interest, therefore, in uncovering subtype or tissue specific synthetic LXR agonists that could mitigate the negative effect of liver LXRα activation. A significant body of work has now identified the LXR subtype and tissue

specific contributions to the anti-atherogenic activity of LXR agonists (see tables 1.1 and 1.2). Yet, LXR activation has pleiotropic effects and can either repress or activate various gene networks. The contribution of these various LXR-dependent pathways to atherosclerosis and the anti-atherogenic effects of agonist treatment are currently under investigation. Since early discoveries identifying a major role for LXRs in regulating macrophage cholesterol efflux and RCT the field has widely believed that these effects underlie much of the athero-protective effects of LXR agonists.

In Chapter Two I investigated the tissue specific contribution of LXR to agonist-stimulated RCT. I chose this project primarily for two reasons – first, despite intriguing findings in the literature[561, 648, 662, 701], I felt that the contribution of LXR in the macrophage, liver and intestine to agonist-stimulated RCT had not yet been systematically addressed. Second, my studies from the JCI manuscript revealed surprisingly that agonist-stimulated macrophage efflux was impaired in *Ldlr*$^{-/-}$/LivKO animals. This unexpected finding led me to question the contribution of LXRs in sites other than the macrophage that may influence agonist-stimulated efflux. My goal for the project was not only to uncover the role of LXRs at various sites on mediating RCT, but also to further the understanding of the potential anti-atherogenic effects of LXR agonists in macrophages. For instance, the atheroprotective activity of LXR agonists requires macrophage LXR expression[549]; however, the contribution(s) of LXR-stimulated cholesterol efflux and LXR-dependent anti-inflammatory activity to this effect is unknown. If my studies revealed that macrophage efflux occurred independently of macrophage LXR expression that would provide evidence that the athero-protective function of LXR in these cells was perhaps due to its anti-inflammatory effects. In the studies reported in Chapter Two, I combined *in vitro* and *in vivo* measurements of cholesterol efflux and RCT with different LXR genetic models to address the role of LXR in the macrophage, liver, and intestine.

Role of Macrophage LXR in agonist-stimulated RCT

The first data I gathered implicated that macrophage LXR activity was neither necessary nor sufficient for LXR agonists to increase either macrophage cholesterol efflux or fecal excretion *in vivo*. In this study I showed that when macrophages from *Lxrα*$^{-/-}$ /*Lxrβ*$^{-/-}$ (DKO) mice were introduced into C57bl6/J animals there was no impairment in agonist-stimulated RCT, at least as measured in an acute assay over a 48 hour time course. This was a somewhat surprising finding as it has been assumed that LXR agonists must act directly on the macrophage to increase macrophage cholesterol efflux. Moreover, there was no agonist-stimulated RCT when C57bl6/J macrophages were introduced into DKO mice. To convince myself that the increase in macrophage cholesterol efflux was a cell autonomous effect, I analyzed gene expression in cells re-extracted from the peritoneal cavity. These studies revealed that indeed the injected macrophages were behaving as expected – C57bl6/J macrophages responded to T0901317 treatment, increasing ABCA1 transcript levels while DKO macrophages did not. To further address the contribution of macrophage LXR to agonist-stimulated macrophage efflux I repeated the *in vivo* RCT study with vehicle and T0901317 treated C57bl/J mice injected with ^{3}H-labeled DKO or LXR+ BMM. I then quantified ^{3}H-cholesterol accumulation in the plasma at time points up to 90 minutes so I could more specifically capture the contribution of the macrophage genotype to agonist-stimulated efflux. Pretreatment with T0901317 significantly increased ^{3}H-cholesterol in the plasma by 60 minutes and macrophage genotype had no effect on the response to agonist. Taken together, these studies show that LXR agonists increase macrophage cholesterol efflux and RCT independently of macrophage LXR activity.

Future Directions: LXR activation in hematopoietic cells is required for the anti-atherogenic activity of LXR agonists[549]; however, data from my studies suggest that this athero-protective function does not arise from macrophage LXR stimulated cholesterol efflux. In addition to increasing expression the of genes involved in cholesterol transport and metabolism, LXRs

agonists are known to have anti-inflammatory activities, primarily by suppressing NFkB-dependent signaling. Inflammation is now widely recognized to be a major contributor to atherogenesis, thus the athero-protective effects of hematopoietic LXRs may reside in their ability to suppress inflammation in macrophages located in atherosclerotic lesions. A model has been proposed suggesting that LXRs repress inflammation through a SUMOylation dependent mechanism that does not require the activation of LXR target genes. Therefore, it may be possible to separate the anti-inflammatory properties of LXR agonists from their cholesterol efflux activities. Genetic and/or pharmacological approaches to address the pathway selective (anti-inflammatory vs. cholesterol efflux) contribution of macrophage LXR to the athero-protective activity of LXR agonists will not only further the understanding of the role of LXRs in atherosclerosis but may lead to the development of novel LXR targeted therapeutics for the treatment of cardiovascular disease. A future study to address how pathway specific LXR activity limits atherosclerosis was the subject of my F30 and AHA fellowship application, which I have included as appendix 1.

Role of intestinal LXR in agonist-stimulated RCT

The finding that LXR agonists increase macrophage efflux independently of macrophage LXR activity led me to question the effect of agonists on the cholesterol acceptor activity of plasma. Previous studies have determined that LXR agonists increase HDL cholesterol by inducing ABCA1 expression in the intestine[529, 579, 661]. Consistent with a potential role for intestinal LXR in regulating agonist-stimulated RCT, pharmacological[648] or genetic approaches[662] selectively activating LXRs in the intestine increases RCT when measured using similar assays to what I used in these studies. HDL-C levels increased in T0901317 treated LXR+ mice and consequently plasma from these animals had increased cholesterol acceptor activity *in vitro*. The effect of agonist was lost however when plasma from DKO animals was used. To further address the contribution of LXR agonist to HDL functional activity, I repeated the *in vitro* efflux experiments using FPLC-purified HDL particles. Using APOA1 as a relative measure of

particle number, I discovered that LXR agonists increase the cholesterol acceptor activity of HDL from LXR+ mice. There was no effect of agonist treatment on the acceptor activity of HDL isolated from DKO animals. Together, these studies demonstrate that LXR agonist treatment not only increases HDL levels, which has been known, but can also promote HDL function, which is a novel finding. One possible explanation is that intestinal LXR activation specifically increases the production of immature nascent particles that are thought to be preferred cholesterol acceptors[663-665].

Over the course of the *in vivo* RCT experiment it is likely that macrophage-derived ^{3}H-cholesterol incorporates into cells and tissues throughout the body. Indeed, approximately 20% or less of injected ^{3}H tracer is recovered in the plasma, liver, feces and re-extracted cells combined at the conclusion of the RCT studies. To address this, as well as the possibility that LXR agonists may increase the amount of cholesterol in plasma by promoting efflux from other sites, I quantified ^{3}H-sterol levels in additional tissues of vehicle or T0901317 pre-treated LXR+ mice injected with ^{3}H-labeled LXR+ macrophages. By mass the majority of ^{3}H labeled was incorporated into skeletal muscle. Interestingly, I observed a significant agonist-dependent decrease in ^{3}H-sterol accumulation in white adipose tissue suggesting that fat may also make an important contribution to LXR-stimulated accumulation of cholesterol in plasma and feces. Importantly, the decrease in adipose ^{3}H-sterol levels could result from increased LXR transcriptional activity in fat cells, the improved activity of HDL or both.

Future Direction: Identify the contribution of LXR activity in the adipocyte to LXR agonist-dependent RCT and increases in HDL function.

Adipose tissue is the largest free cholesterol reservoir in the body[702] and abundantly expresses ABCA1[703]. Moreover, there is accumulating evidence that an imbalance in cholesterol levels in adipose tissue results in adipocyte dysfunction and obesity-mediated metabolic complications, including low levels of HDL cholesterol and insulin resistance[704]. Deletion of

ABCA1 specifically in adipocytes reduces apoA1-stimulated cholesterol efflux from fat and decreases nascent HDL particle formation[705]. Furthermore, there is a strong positive correlation between adipocyte cholesterol content and ABCA1 expression[705] suggesting that LXR may play an important role in mediating ABCA1-dependent efflux and HDL metabolism in fat. It is possible, therefore, that an additional site of LXR dependent increases in HDL mass and function arises from activation in adipocytes; a possibility that was not explored in my studies. Moreover, the observation that LXR agonist treatment prevents high fat diet-induced obesity and insulin resistance in C57bl6/J[706] mice supports the potential importance of adipocyte LXRs. The Collins laboratory published studies of an adipose LXRα knockout in which they found that these mice gained more weight and fat mass on a high-fat diet indicating that LXRα plays an important role in adipocyte lipolysis and fatty acid oxidation[707]. While the adipose-specific LXRα knockout and global *Lxrα*$^{-/-}$ animals are obesity prone[708], *LXRα*$^{-/-}$ /*LXRβ*$^{-/-}$ animals have been shown to be obesity resistant[572, 709], suggesting that LXRβ may mediate lipolysis and energy balance in adipose as well. Thus, a complete adipose double *Lxrα*$^{-/-}$ /*Lxrβ*$^{-/-}$ knockout should be generated in order to address the contribution of LXRs in the adipose to RCT and HDL metabolism. The adipose-specific LXRα animals could be crossed into the global LXRβ$^{-/-}$ knockout to generate adipose-specific *Lxrα*$^{-/-}$ *Lxrβ*$^{-/-}$ animals. An alternative approach, although less favorable, would be to use the adipocyte-specific *Abca1*$^{-/-}$ knockout mouse as LXR regulated cholesterol efflux and effects on HDL synthesis would likely be greatly diminished in these animals as well. Measuring LXR agonist-stimulated cholesterol efflux *in vivo* and *in vitro* in an adipocyte-specific *LXRα*$^{-/-}$ /*LXRβ*$^{-/-}$ knockout animal or alternatively the adipose-specific *Abca1*$^{-/-}$ would address the contribution of LXR activity in the fat to RCT and HDL.

CETP inhibits LXR agonist-stimulated macrophage efflux

To test the hypothesis that agonist-dependent increases in HDL levels are responsible for stimulating macrophage efflux in T0901317 treated animals, I took advantage of the CETP transgenic model in which LXR agonists lose the ability to raise HDL-cholesterol[598, 657]. In my

studies LXR agonist treatment increased CETP expression and plasma activity and consequently lowered HDL levels in these animals. Both *in vivo* agonist-dependent macrophage cholesterol efflux as well as the acceptor activity of FPLC-purified HDL was decreased in CETP transgenic mice. These findings supported the hypothesis that the macrophage cholesterol efflux measured in the *in vivo* RCT assay is primarily determined by the amount and functional activity of HDL. The finding that increasing CETP activity impairs HDL function is consistent with reports that inhibition of CETP activity improves the cholesterol acceptor activity of human HDL particles[667].

Despite a loss of agonist-stimulated macrophage cholesterol efflux *in vivo*, I found that LXR agonist treatment still increased the fecal excretion of macrophage-derived cholesterol in the CETP transgenic mice. Using adeno-viral over expression Tanigawa et al.[710] also observed a similar ability of CETP expression to increase fecal cholesterol excretion without impacting macrophage efflux. Taken together, I believe that these results suggest, at least under these conditions, that macrophage cholesterol efflux is not a rate-limiting step for RCT. Combined with the finding that fecal cholesterol excretion is controlled by liver LXRα activity, these studies indicate that it is possible to functionally segregate macrophage cholesterol efflux from fecal excretion. Macrophage efflux is primarily controlled by the ability of LXRs to increase the quantity and quality of HDL while fecal excretion is controlled by LXR-dependent regulation of hepatic ABCG5 and ABCG8 levels allowing LXRs to coordinate cholesterol movement throughout the body.

The effect of diet on Liver LXRα activity

In the absence of liver LXRα expression T0901317 fails to increase the hepatic expression of genes involved in cholesterol excretion (ABCG5 and ABCG8) and bile acid synthesis (CYP7a) and consequently agonist-stimulated fecal cholesterol excretion is lost. On standard chow diet deletion of LXRα in the liver has no effect on the ability of LXR agonists to increase intestinal ABCA1 expression, raise HDL-C levels and HDL acceptor function or to promote macrophage efflux. However, when challenged with a 0.2% cholesterol diet LXR

agonist-dependent macrophage cholesterol efflux is significantly impaired. Consistent with the loss of agonist-stimulated macrophage efflux, the ability of agonists to increase HDL levels and function is also significantly attenuated 0.2% cholesterol diet fed LivKO animals. From my studies I was unable to determine the reason(s) for the loss of the positive effect of agonists on HDL in LivKO mice under dietary cholesterol challenge. LXR agonist treatment still increased ABCA1 expression in the intestine of 0.2% cholesterol diet fed LivKO animals so presumably HDL levels should have similarly increased. Compared to littermate controls on the 0.2% cholesterol diet, LivKO mice had increased hepatic cholesterol levels although I did not detect any evidence for increased hepatic inflammation, endoplasmic reticulum stress or liver damage that might influence HDL formation. We and others have shown, however, that the ability of LXR agonists to increase HDL levels is lost under severe hyperlipidemic conditions such as *Ldlr*$^{-/-}$ or *Apoe*$^{-/-}$ mice on Western diets[529, 548, 552, 628, 647]. Thus, the ability of LXR agonists to regulate HDL levels may in fact be influenced by the amount of dietary cholesterol present. Interestingly, *Lxrα*$^{-/-}$/*Lxrβ*$^{-/-}$ are resistant to high fat diet-induced obesity, however, this resistance is only observed when the high fat diet also contains cholesterol[572].

Future Directions – Identification of cholesterol sensitive signaling molecules in LivKOs

Hepatic cholesterol increases in LivKO animals fed cholesterol enriched diet compared to littermate controls. This accumulation of hepatic cholesterol may lead to the generation of a paracrine or endocrine signal that could alter lipid metabolism in other tissues thereby influencing LXR-dependent HDL metabolism. In support of the potential role of liver LXRα in regulating hepatic endocrine signaling, bile acids, in addition to their well-established roles in cholesterol homeostasis, also have systemic endocrine functions[711]. Bile acids have been shown to activate mitogen-activated protein kinase (MAPK) pathways[712], G protein coupled receptors[713], and the farnesoid X receptor (FXR)α[714]. Through activation of these signaling pathways, bile acids can regulate triglyceride, cholesterol, and energy and glucose homeostasis. Thus, bile acids may be an

important modulator of lipoprotein metabolism. Bile acids are the endogenous ligands for FXRα and as such FXRα is abundantly expressed in both the liver and intestine[715]. Importantly, several genes with a role in HDL metabolism are FXRα targets. For instance, FXRα induces human and rodent ApoC-II expression[716]. ApoC-II is a coactivator of lipoprotein lipase and its induction lowers serum triglycerides. Activation of FXRα in mouse models has been shown to reduce ApoA1 expression and lowers HDL-C levels[717] whereas FXR-deficient mice are hypercholesterolemic because of an increase in HDL-C[718]. Polyunsaturated fatty acids (PUFA) like arachidonic and linolenic acid[719] as well as intermediates of the bile acid synthesis pathway[720] have been shown to be FXR ligands and modulators *in vitro* Bile acid intermediates have been proposed to be important FXR ligands during cholestasis or metabolic disorders when the compounds could potentially be in abundance.

In the absence of liver LXRα activity bile acid synthesis is greatly reduced[529] largely due to the loss of LXR-dependent increases in CYP7a expression. CYP7a is the rate-limiting step of the bile acid biosynthetic pathway. In the absence of CYP7a activity, it is possible that additional enzymes compensate and result in an accumulation of bile acid intermediates. Thus, in the setting of increased hepatic cholesterol such as occurs in 0.2% cholesterol diet fed LivKO mice, there is could be an accumulation of bile acid intermediates that activate FXR. In addition, liver LXRα deletion could increase PUFA synthesis similar to what has been reported in NCOR deficient macrophages due to the loss of LXR repressing activity[614]. Taken together, the potential increase in PUFAs and bile acid intermediates that occurs in 0.2% cholesterol diet fed LivKO mice that could activate FXRs and subsequently lower HDL levels and function. This possibility could be explored in future studies, perhaps by using adeno-viral delivery of siRNAs targeted to FXR and/or pharmacologically inhibiting FXR activity in 0.2% cholesterol diet fed LivKO mice. The expectation would be that inhibiting FXR would restore LXR agonist-stimulated increases in HDL mass and function in cholesterol diet fed LivKO mice.

LXR agonists enhance HDL cholesterol acceptor function

Recent clinical trials with niacin[633] and CETP[428] inhibitors have raised serious doubts about the cardio-protective effects of raising HDL cholesterol. 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 suggests that assessing HDL function may be more relevant than measurements of HDL cholesterol[371, 381, 412]. In addition to increasing HDL cholesterol levels, my studies presented in chapters two and three provide evidence, for the first time, that LXR agonist treatment also increases the cholesterol acceptor activity of HDL particles. HDL particles are heterogeneous in size and composition making it difficult to discern the LXR-dependent modifications that improve cholesterol acceptor activity. Yet, upon initial analysis I found that LXR agonist treatment increased the HDL phospholipid levels (normalized to ApoA1). The phospholipid:ApoA1 ratio in HDL is an important determining factor in predicting macrophage efflux[483, 484] and studies with human sera show that a the correlation between macrophage efflux and HDL phospholipid levels is stronger than with any other measured lipoprotein parameter, including HDL cholesterol, APOA1 and triglycerides[485].

The ability of LXR agonists to increase the acceptor capacity of HDL particles is lost in both 0.2% cholesterol-diet challenged LivKO and hyperlipidemic *Ldlr*$^{-/-}$ /LivKO animals as well as in the presence of CETP expression. In the cholesterol-fed LivKO animals the loss of agonist-stimulated HDL function also correlates with an inability of LXR agonist treatment to increase HDL-phospholipid levels (HDL-phospholipids weren't measured in *Ldlr*$^{-/-}$ /LivKO animals). Why agonist treatment fails to promote HDL-phospholipids in cholesterol-diet fed LivKO animals may be, a suggested above, due to FXR activation by bile acid intermediates or PUFA. CETP expression, however, impacts HDL function without modulating phospholipid levels suggesting that multiple components of HDL can influence particle function. Furthermore, additional studies in our lab identified changes in HDL associated peptides (data not shown) in hyperlipidemic *Ldlr*$^{-}$

$^{-/-}$ /LivKO compared to floxed controls further providing evidence that LXRs can regulate HDL lipids and proteins.

Future Directions: High resolution proteomic and lipidomic analysis of lipoprotein particles from LivKO mice.

LXRs likely regulate multiple pathways that modulate HDL activity and future studies using detailed proteomic and lipidomic approaches could be used to further define the LXR-dependent changes in HDL composition that regulate HDL particle function. Such studies that define the LXR-agonist dependent changes in particle composition that are associated with increased particle function (cholesterol acceptor activity) would be insightful for future HDL-targeted therapeutic strategies. Proteomic and lipidomic analysis of lipoprotein particles is still in its infancy and results differ greatly depending on particle isolation procedures. The majority of studies have used ultracentrifugation to isolate HDL prior to proteomic analysis, however this method does not provide complete separation from either plasma proteins or LDL[721, 722]. Importantly, populations of small LDL particles in particular have been shown to co-precipitate with HDL_2 particles upon ultracentrifugation[722]. My studies described in chapter three identified a preponderance of small LDL in plasma from hyperlipidemic *Ldlr*$^{-/-}$ /LivKO mice; therefore, any future studies examining LXR-dependent changes in HDL composition and function should employ high resolution FPLC and not ultracentrifugation.

Contribution of Liver LXRα to HDL function and Atherosclerosis

Studies presented in Chapter Three characterize the liver-specific LXRα knockout mouse, the first conditional LXR-deficient animal, which was generated by the Mangelsdorf laboratory at UT-Southwestern. Members of the Mangelsdorf lab provided the early work that demonstrated the critical role for liver LXRα in regulating hepatic cholesterol excretion and catabolism. Several studies have described a *trans*-intestinal pathway for cholesterol excretion independent of the biliary system that can be stimulated by LXR activation[577, 685, 686]. The work from Mangelsdorf's

group, however, suggests that the trans-intestinal pathway makes only a minor contribution to LXR agonist-dependent cholesterol excretion.

Synthetic LXR agonists increase plasma triglycerides and plasma HDL cholesterol, however, which sites contributed to these pharmacological responses to LXR activation were unknown[524, 583]. Analysis of the LivKO knockout mice demonstrated that these responses occur at unique sites. LXR activation in the liver is responsible for agonist-dependent increases in plasma triglycerides while LXR activation in the intestine drives the agonist-stimulated rise in HDL cholesterol. Indeed, consistent with the idea that intestinal LXR activity is primarily responsible for elevating HDL cholesterol, Brunham et al[579] showed that expression of ABCA1 in the intestine is required for LXR agonist-dependent HDL cholesterol increases, while Lo Sasso et al. demonstrated that transgenic overexpression of a constitutively active LXRα in the intestine increases HDL[662].

Treatment with LXR agonists is athero-protective in mouse models, however, in these hyperlipidemic settings LXR agonists have little or no effect on HDL cholesterol levels, and this has led to the conclusion that the anti-atherogenic activity originates from increased macrophage cholesterol efflux and/or other LXR regulated pathways in immune cells in atherosclerotic plaques[548, 549, 628]. Yet, the anti-atherogenic activity of LXRs is not solely derived from activation in immune cells. While selective deletion of LXRα in hematopoietic cells increased atherosclerosis (*Ldlr*$^{-/-}$ background), the effect was not as great as measured in the *Ldlr*$^{-/-}$ */Lxrα*$^{-/-}$ global knockout mice[549, 569, 628]. Therefore, following the initial characterization of the LivKO animals performed by Mangelsdorf's group, we generated *Ldlr*$^{-/-}$/LivKO animals in order to address to the role of liver LXR in atherosclerosis. Deletion of LXRα in hepatocytes increases Western diet induced atherosclerosis, indicating that the liver is a critical site of LXRα-dependent athero-protective activity.

Noting striking differences in the plasma lipid levels between Ldlr$^{-/-}$/LivKO mice and *Ldlr*$^{-/-}$/floxed controls, I analyzed the lipoprotein profiles of these mice looking for any change in

particle number or composition that might influence the increased atherogenicity of the *Ldlr*$^{-/-}$/LivKO mice. My analysis suggested that hepatic LXRα modulates lipoprotein particle number, size, and function in a manner that influences atherosclerosis. As I describe in the studies presented in Chapter Three, there is impairment in HDL cholesterol acceptor function in hyperlipidemic *Ldlr*$^{-/-}$/LivKO mice. Impaired HDL function in these animals correlated with a decrease in total HDL particle number that was largely the result of a decrease in small HDL (diameter <8.2 nm). Furthermore, while *Ldlr*$^{-/-}$/floxed and *Ldlr*$^{-/-}$/LivKO animals had similar numbers of LDL particles, there was a dramatic shift in particle size with almost 50% of the *Ldlr*$^{-/-}$/LivKO particles being small LDL (diameter < 21 nm). As discussed in Chapter One, small LDL particles are believed to have increased atherogenic properties and promote macrophage foam cell formation. Yet I did not detect a difference in cholesterol accumulation when bone marrow-derived macrophages were cultured *in vitro* in the presence of plasma or FPLC-purified apoB-containing lipoproteins from *Ldlr*$^{-/-}$/floxed or *Ldlr*$^{-/-}$/LivKO animals. Furthermore, I did not detect any differences in either the oxidative properties or inflammatory state of plasma or FPLC-purified apoB-containing lipoproteins from *Ldlr*$^{-/-}$/floxed or *Ldlr*$^{-/-}$/LivKO animals. Thus, preliminary analysis of apoB-containing lipoproteins from *Ldlr*$^{-/-}$/LivKO animals suggests that despite the change in size there is no increase in atherogenicity of these particles. More detailed studies, however, including lipidomic and proteomic analysis of *Ldlr*$^{-/-}$/LivKO LDL and HDL particles, similar to the proposal outlined above, would provide valuable insight and address such questions as the contribution of different pathways to atherogenesis (i.e. HDL function vs. LDL atherogenicity) as well as the role of hepatic LXRα in atherosclerosis.

Despite the increase in atherosclerosis in *Ldlr*$^{-/-}$/LivKO animals, agonist treatment still effectively reduces disease, indicating that the anti-atherogenic activity of LXR agonists is extra-hepatic. My *in vivo* RCT analysis in *Ldlr*$^{-/-}$/LivKO mice indicates that fecal cholesterol excretion is not required for the protective action of agonists. Intriguingly, I discovered that agonist-stimulated macrophage efflux also appeared to be blocked in *Ldlr*$^{-/-}$/LivKO thereby suggesting

that macrophage efflux was also was not required for the cardio-protective effects of LXR agonsits. This observation led to the studies presented in Chapter two as well as the hypothesis that an additional LXR regulate pathway in immune cells is responsible for the anti-atherogenic activity of agonists. A number of additional functions for LXRs in immune cells have been identified, including the ability to control inflammation[640], endoplasmic reticulum stress[695], macrophage egress[556], and monocyte proliferation[697, 698]. Any one or a combination of these activities could be responsible for providing athero-protection in response to macrophage LXR activation by agonists. I believe a major path forward from my body of work would be the identification of such a pathway(s) in macrophages that are responsible for the athero-protective activity of LXR agonists. This future direction is described in detail in the following chapter.

In conclusion, the novel findings from this body of work include:

1. Macrophage LXR activity is neither necessary nor sufficient to promote agonist stimulated RCT.
2. Liver LXRα regulates LXR-dependent lipogenesis, biliary cholesterol excretion and fecal cholesterol loss.
3. LXR-dependent increases in HDL-cholesterol originate from LXR regulation of ABCA1 in the intestine.
4. Agonist-dependent increases in HDL levels and cholesterol acceptor activity drives agonist-stimulated macrophage cholesterol efflux *in vitro* and *in vivo*.
5. In the presence of CETP, the ability of LXR agonists to increase HDL quantity and quality is lost and consequently agonist-stimulated macrophage efflux is impaired.
6. On a chow diet, there is no effect of liver LXRα deletion on HDL function and macrophage cholesterol efflux *in vivo*; however, the presence of dietary cholesterol impairs agonist-stimulated increases in HDL quality and quantity and macrophage efflux in liver-specific LXRα deficient mice.

7. There is a change in lipoprotein particle number and size in *Ldlr*$^{-/-}$/LivKO animals reflecting a preponderance of small LDL and reduction in small HDL.
8. Loss of hepatic LXRα activity increases atherosclerosis.
9. The atheroprotective function of LXR agonists is independent of hepatic LXRα activity and does not require an increase in fecal cholesterol excretion; furthermore, the protective function of LXR agonist may even be independent of increased macrophage cholesterol efflux.

APPENDIX 1: Pathway specific LXR activity limits atherosclerosis

What lies herein is a version of a grant application submitted in late 2012 for consideration for a F30 fellowship from the NIHLBI. This proposal received an impact score of 20 however was unfunded. I believe that this proposal presents a relevant path forward in continuation of the novel findings that have arisen from my body of work. In the time since this proposal was submitted, however, an alternative mechanism for LXR anti-inflammatory activities has been published which complicates my proposed project below. I have address this new data and presented an additional method to study the contribution of macrophage LXR anti-inflammatory activity to the athero-protective function of LXR agonists at the conclusion of this chapter.

1. SPECIFIC AIMS

Atherosclerosis, one of the greatest health concerns of the western world, is a disease marked by chronic inflammation and disordered lipid metabolism. The Liver X Receptors, LXRα (NR1H3) and LXRβ (NR1H2) are ligand activated transcription factors that control cholesterol homeostasis and suppress inflammation throughout the body. Treatment with LXR agonists has been shown to reduce atherosclerosis in animal models, an effect dependent on macrophage LXR expression. A major limitation to the development of LXR ligands for the treatment of cardiovascular disease, however, is their propensity to increase plasma triglycerides by inducing sterol regulatory element binding protein 1c (SREBP1c) mediated hepatic lipogenesis.

LXR activation increases the expression of genes regulating reverse cholesterol transport (RCT), the movement of cholesterol from the periphery through the liver ultimately promoting increased fecal cholesterol excretion. LXR regulates RCT through transactivation (Figure 5.1A), or the agonist dependent recruitment of co-activator proteins to the promoter of target genes. Recent studies also have identified an important role for LXR in suppressing inflammatory genes such as interleukin β (IL-1β), Inducible Nitric Oxide Synthase (iNOS), and Monocyte chemoattractant protein 1 (MCP-1), which all contribute to atherogenesis (Figure 6.1B). Agonist binding promotes a conformational change in LXR allowing receptor SUMOylation. SUMOylated LXR is recognized and bound by corepressor complexes at the promoter region of NFκB pro-inflammatory genes. Upon inflammatory stimulus – e.g. LPS or oxidized LDL – SUMOylated LXR prevents corepresssor dissociation thereby maintaining active gene repression. LXR agonists that have demonstrated the atheroprotective activity of LXRs are not pathway selective, therefore, it is unclear whether LXR transactivation of metabolic/RCT genes (Figure 5.1) or LXR transrepression (Figure 1B) of inflammatory signaling, or a combination of both is responsible for the beneficial effects of synthetic agonists.

We have recently shown that the athero-protective activity of LXR agonists is independent of promoting RCT and, furthermore, increased macrophage cholesterol efflux may not even be required. However, LXR expression is necessary in the hematopoietic compartment for the beneficial effect of agonists. Therefore, we hypothesize that transrepression by LXR of inflammatory signaling in the macrophage is responsible for the anti-atherogenic effect of synthetic LXR agonists. First generation LXR agonists have failed in the clinic because of their propensity to increase plasma triglycerides due to LXR transactivation in the liver. However, if transrepression is responsible for the beneficial activity of LXRs, a 2nd generation of pathway selective LXR agonists could be developed that would circumvent the negative side effects associated with 1st generation agents. **The main goal of this proposal is to determine the contribution of anti-inflammatory LXR activity to the athero-protective effect of LXR agonists**.

SPECIFIC AIM 1: Identify and characterize LXR mutants that dissociate LXR transactivation and transrepression pathways. It has recently been suggested that the transrepression of inflammatory signals by LXR agonists is SUMOylation dependent, and LXR SUMOylation defective mutants inhibit LXR transrepression, however, have little to no effect on transactivation. In Aim 1 we will use site directed mutagenesis to identify LXR mutants that dissociate LXR transactivation from LXR transrepression, and these mutants will be characterized in mouse primary macrophages.

SPECIFIC AIM 2: **Determine the contribution of anti-inflammatory LXR activity to the athero-protective effect of LXR agonists**. LXR agonists are athero-protective in the absence of RCT, thereby suggesting that the beneficial effect of agonists may be due to the anti-inflammatory properties of LXR in macrophages. $LDLR^{-/-}$mice will be irradiated and reconstituted with bone marrow from transgenic mice that express pathway selective LXR mutants – i.e. promote macrophage cholesterol efflux or prevent inflammatory signaling. Recipient $Ldlr^{-/-}$ mice will be fed a western diet in the absence or presence of LXR ligand and at the end of the study the extent of atherosclerosis will be quantified. This study will provide fundamental knowledge concerning the pathogenesis of atherosclerosis (e.g. inflammation vs. cholesterol accumulation) thereby identifying the important mechanism to target for the treatment of cardiovascular disease.

2. BACKGROUND AND SIGNIFICANCE

Atherosclerosis

Critical to the development of atherosclerosis is the unregulated accumulation of oxidized cholesterol by macrophages in the blood vessel wall and the associated inflammatory response that leads to foam cell formation[723]. The role for increased plasma lipids and inflammation in the initiation and progression of atherosclerosis is now well appreciated[723-725]. Despite the efficacy of current therapy for lowering plasma lipids, the residual risk for developing complications from cardiovascular disease remains at ~75% in these patients[726]; thus alternative therapies, such as promoting the removal of cholesterol from the macrophage or limiting the inflammatory response are being explored[727].

LXRs and Macrophage Reverse Cholesterol Transport

The LXR sub-group of the nuclear hormone receptor superfamily is comprised of two subtypes, LXRα and LXRβ[512]. The two subtypes have considerable sequence homology; however, they differ in tissue expression. LXRα is more highly expressed in the liver, kidney, intestine and macrophages. In contrast, LXRβ is more ubiquitously expressed[516]. Both LXRs bind to DNA and regulate transcription as heterodimers with retinoid X receptors (RXRs)[518]. The link between LXRs and lipid metabolism came from the identification of cholesterol derivatives including 22(R)-hydroxycholesterol, 24(S)-hydroxycholesterol, and 24(S),25-hydroxycholesterol as agonists that directly bind to both LXRs and increase their transcriptional activity by promoting the release of trans-acting corepressor and interactions with trans-acting coactivators[520, 548, 728-730].

Gene expression analysis of mice treated with synthetic LXR agonists identified the ATP binding cassette transporter ABCA1 as a direct LXR target gene[731, 732]. ABCA1 is required for the process of reverse cholesterol transport (RCT), the mechanism by which peripheral cells efflux internal cholesterol to HDL particles[380, 733]. Loss of functional ABCA1 results in Tangier disease, a condition in which patients have low levels of circulating HDL and an increased risk for developing atherosclerosis. Fibroblasts from patients with Tangier disease are unable to efflux cholesterol, suggesting that low HDL levels and increased risk of atherosclerosis results from loss of reverse cholesterol transport[376-378]. As described above, accumulation of oxidized cholesterol by macrophages in the arterial wall is an initiating step in the development of atherosclerotic lesions. Not surprisingly, transfer of ABCA1 deficient macrophages into hyperlipidemic mice results in increased levels of atherosclerosis[734].

Treatment of primary macrophages with LXR agonists results in induction of the ABCA1 gene, increased ABCA1 protein and an increase in cholesterol efflux[732]. Importantly, binding sites for LXR-RXR heterodimers have been identified in the promoter of the ABCA1[732] gene and in the control regions of other genes encoding additional proteins involved in reverse cholesterol transport, such as ABCG1[735]; thus, activation of LXR promotes a mobilization of cellular cholesterol from peripheral macrophages and other cells to HDL[562].

Regulation of Hepatic Lipid Metabolism by LXR

LXRα single knockout mice accumulate large amounts of cholesterol in the liver when challenged with a high cholesterol diet. Molecular analysis uncovered aberrant regulation of CYP7a, which encodes cholesterol 7α hydroxylase, the rate-limiting enzyme in the conversion of cholesterol to bile acids[520, 541]. Additionally, the ATP binding cassette transporters ABCG5 and ABCG8 which excrete cholesterol out of the liver into the intestine were identified as LXR target genes[646]. Therefore in the liver LXRs control the catabolism of cholesterol to bile acids and the excretion of cholesterol into the intestine. Combined with the effects on reverse cholesterol transport in peripheral cells, activation of LXR results in the mobilization of cholesterol from the periphery and elimination from the body via catabolism and excretion. LXRs, therefore, monitor overall cholesterol balance by controlling the initial steps of RCT (transfer to HDL) in peripheral cells and the final steps of RCT (catabolism and excretion) in the liver[541, 561].

Along with effects on cholesterol homeostasis, LXRs regulate expression of genes involved in fatty acid metabolism including the master transcriptional regulator of fatty acid synthesis SREBP1c[583, 584], fatty acid synthase (FAS)[524], and stearoyl CoA desaturase (SCD-1)[524]. The up-regulation of fatty acid synthesis is suggested to provide lipids for the storage of cholesterol as cholesterol esters. Indeed, treatment of mice with synthetic LXR ligands results in an increase in plasma triglycerides[529, 552, 736].

Anti-Inflammatory Activity of LXR

In addition to stimulating reverse cholesterol transport, studies in macrophages indicate that LXRs can inhibit the expression of several pro-inflammatory genes including iNOS, COX-2, and MMP-9[603]. Additionally, LXR agonists are effective in a mouse model of contact dermatitis[671]. Molecular analysis indicates that activation of LXR decreases the transcriptional activity of NFκB[737]. Since inflammation plays an important role in the pathogenesis of atherosclerosis the questions remains whether LXR mediates its anti-atherogenic activity via control of reverse cholesterol transport, by limiting the inflammatory response, or both. A pathway involving agonist-dependent SUMOylation of LXR appears necessary for the repression of inflammatory gene expression[1, 613]. Importantly, transcriptional repression by LXRs is mechanistically distinct from the positive activation of genes involved in RCT; thus suggesting that these two LXR activities can be dissociated. Indeed, GW9772, a synthetic LXR ligand, has been reported to dissociate LXR activity in vitro; however, the poor bioavailability of GW9772 has prevented it from being tested *in vivo*[628].

3. PRELIMINARY DATA

RCT is inhibited in the absence of liver LXRα activity.

To examine the contribution of hepatic LXR activity to the RCT pathway we crossed liver-specific LXRα knockout (LXRα$^{floxed/floxed}$ + albumin-CRE^{+}) into the *Ldlr*$^{-/-}$ background to create *Ldlr*$^{-/-}$ /Lxrα$^{-/-}$ double knockout mice (referred to as *Ldlr*$^{-/-}$ /LivKO). Importantly LXRα is the major LXR subtype expressed in the liver[512]. RCT was measured *in vivo* using the assay developed by Rader and colleagues[561]. Briefly, mouse J774 macrophages were loaded with ^{3}H-cholesterol and acetylated LDL *in vitro* and then injected into the peritoneal cavity of *Ldlr*$^{-/-}$ /LivKO and albumin-CRE negative littermate controls (referred to as *Ldlr*$^{-/-}$ /Floxed) that had been on western diet for 9 weeks in the absence or presence of the LXR agonist T0901317. The amount of ^{3}H tracer appearing in the plasma, liver and feces was determined 48 hours later. As expected, agonist-stimulated fecal cholesterol excretion was lost in the absence of liver LXRα[529]. Somewhat more surprising, however, was the finding that the appearance of ^{3}H tracer in the plasma was significantly reduced and unresponsive to LXR agonists (Figure 3.8). The ability of LXR agonists to increase the appearance of macrophage-derived ^{3}H-cholesterol in the plasma is thought to result from agonists acting on macrophage LXRs to enhance ABCA1 dependent cholesterol efflux[562]. To determine if the decrease in plasma ^{3}H-cholesterol levels observed in *Ldlr*$^{-/-}$ /LivKO animals resulted from impaired LXR transcriptional activity in macrophages, we recovered the J774 cells from the peritoneal cavity 48 hours following injection and quantitated ABCA1 mRNA levels. Agonist treatment produced a similar increase in ABCA1 mRNA in cells recovered from either *Ldlr*$^{-/-}$ /Floxed or *Ldlr*$^{-/-}$ /LivKO animals[529]. The gene expression analysis suggests that the failure of LXR agonist to increase the appearance of macrophage-derived ^{3}H-cholesterol in the plasma of *Ldlr*$^{-/-}$ /LivKO mice does not arise from a defect in macrophage LXR activity. Additional studies suggest that the production of defective HDL particles in the LivKO may account for this defect in macrophage cholesterol efflux[529].

LXR agonist maintains anti-atherogenic activity independent of Liver LXR expression

Liver-specific knockout of LXRα in the *Ldlr*$^{-/-}$ background significantly increases atherosclerosis (Figure 3.6), however, treatment with T0901317 is still able to significantly reduce atherosclerosis in the *Ldlr*$^{-/-}$ /LivKO animals (Figure 3.6). Importantly, the magnitude of agonist-dependent reduction of atherosclerosis lesion size was similar to that observed in the control mice (Figure 3.6). These findings suggest that LXR agonists can reduce atherosclerosis independent of promoting RCT. Since previous studies from our lab suggest that hematopoietic LXRα activity is required for the anti-atherogenic activity of LXR agonists[549] (note that LXRα is restricted to the myeloid lineage in hematopoietic cells[512]), we hypothesize that an alternative LXR regulated pathway is responsible for mediating the beneficial effects of LXR agonists in macrophages. One alternative pathway that may contribute significantly to the anti-atherogenic activity of LXR agonists is the inhibition of inflammatory signaling. As mentioned previously, the anti-inflammatory activity of LXRs is mediated by a process quite distinct from LXR regulated cholesterol efflux (Figure 1); however, current LXR agonists with therapeutic efficacy are not pathway-selective. Therefore, I proposed using LXR pathway selective mutants to determine the relative contribution of LXR stimulated RCT and the LXR dependent inhibition of inflammatory activity to the beneficial activity of agonists in macrophages.

4. RESEARCH DESIGN AND METHODS

AIM 1: Dissociate LXR activity

1.1 Identification of pathway selective LXR mutants

RATIONALE: Preliminary work in our lab suggests that synthetic LXR agonists can maintain anti-atherogenic activity without promoting RCT[529] and that macrophages are a critical site for LXR athero-protective activity[549]. In the macrophage, LXR promotes cholesterol efflux and inhibits inflammation through two independent, ligand-dependent mechanisms (Figure 1). However, the relative contribution of LXR pathway specific activity (i.e. activation vs. repression) has yet to be determined. Towards this goal, we will identify and characterize pathway selective LXR mutants in macrophages.

EXPERIMENTAL DESIGN:

Ligand binding to LXRs initiates a conformational change in the receptor which promotes the exchange of trans-acting co-repressors for co-activators and the subsequent increase in transcriptional activity. This process is termed transactivation and, importantly, is also dependent on LXR-DNA interactions (Figure 6.1A). Conversely, ligand binding also promotes conjugation of LXR with SUMO2/3 by the SUMO-conjugating enzyme (UBC9) in a Histone Deactelyase 4 (HDAC4) dependent manner. SUMOylated LXRs recognize trans-repressing complexes at the promoter region of inflammatory genes and inhibits the release of the complexes that usually occur upon NFκB binding (Figure 6.1B). This process by which LXR actively represses inflammation in the presence of inflammatory signaling is called transrepression. Using site directed mutagenesis, we will make the mutations listed in Table 6.1 in order to dissociate LXR activity. Lysine to arginine mutations at LXRβ at residues 410 and 448, and in human LXRα at residues 328 and 434 abolish receptor sumoylation and accordingly block LXR agonist dependent transrepression without having an effect on LXR transactivation[1]. These findings were recently validated for LXRβ 410/448 in human and mouse hepatocytes[613]. Interaction between sumoylated LXR and Coronin2A, a subunit of the co-repressor complex, is required for agonist dependent transrepression in macrophages[611]; thus, we will also generate a LXRβ S427D mutant that has been shown to be unable to interact with Coronin2A and promote transrepression[611].

The interaction between LXR and coactivator proteins that mediate transactivation has been well characterized[545, 738]. The conformational change that occurs following ligand binding stabilizes helix 12, which is a highly dynamic aliphatic alpha helix located in the C-terminal domain of LXR. A hypdrophobic cleft is created upon stabilization of helix 12 that allows coactivator proteins to bind, thereby promoting the induction of LXR target genes[680]. LXRs contain N-terminal zinc fingers regions that mediate receptor binding to LXR response elements (LXREs)[739]. Binding of receptor to LXREs upstream of target genes is required for transactivation but not for transrepression[1, 613, 730]. Helix 12 and zinc finger mutants are described in Table 1. Based on the crystal structures of the LXR receptors, these transactivation defective mutants (Table 1) should disrupt interaction with coactivator proteins or DNA but should not influence ligand binding or overall protein structure[512, 680]. Indeed, we have preliminary data that the LXRα helix 12 and DBD mutants are transactivation defective (Figure 2B and data not shown). The LXRβ mutants are currently being examined.

To measure agonist-dependent transrepression, the mutants described above will be co-transfected into Hela cells with a luciferase reporter gene containing 3 upstream copies of the NFκB-binding sites from the human IL-8 promoter. Transfected cells will be cultured in the absence or presence of LXR ligand overnight and the following day TNFα or vehicle will be added in the continued presence of LXR ligand to induce NFκB signaling. Six hours after the addition of TNFα, cells will be lysed and luciferase activity quantified. Vehicle treated cells transfected with NFκB-reporter alone and ligand-treated cells transfected with WT LXR will serve as negative and positive control respectively (Figure 6.2A).

To measure agonist-dependent transactivation the mutants described above will be co-transfected into Hela cells with a luciferase reporter gene containing 3 upstream LXREs. Transfected cells will be cultured in the absence or presence of LXR ligand for 24 hrs, cells lysed, and luciferase activity measured (Figure 2). Vehicle treated cells transfected with LXRE reporter alone and ligand treated cells transfected with WT LXR will serve as negative and positive controls, respectively. Data for both assays (transrepression and transactivation) will be analyzed by 1-way ANOVA followed by Dunnett's post-test to determine statistically significant differences among groups.

Mutants that fail to inhibit LPS induced activity but maintain levels of agonist-stimulated gene expression comparable to WT LXR will be considered to be transactivation selective. Similarly, mutants that fail to activate gene transcription but inhibit LPS induced activity comparable to WT LXR following ligand treatment will be considered to be transrepression selective.

EXPECTED RESULT: We expect that mutating the sumoylated lysine residues in the ligand-binding domain of the LXRs will disrupt agonist dependent transrepression without impacting transactivation. Furthermore, we anticipate that our helix 12 and zinc finger mutants dissociate either coactivator or DNA binding without effecting sumoylation thereby generating transrepression selective mutants. A single representative of each class of mutant (transactivation defective mutant and transrepression defective mutant), regardless of subtype, that demonstrates pathway selectivity will be used in the studies proposed in experiment 1.2.

POTENTIAL PROBLEMS AND ALTERNATIVE APPROACHES: Three other additional sites of sumoylation have recently been reported for LXRβ – K30, K395 and K433. If the SUMO mutants described above fail to behave as expected we will examine the activity of these additional SUMO mutants. Since sumo modification is required for LXR transrepression[1, 613], we believe that one or a combination of these additional sumoylation mutations will disrupt LXR transrepression.

Preliminary data in our lab indicate that LXR Helix 12 and zinc finger mutants fail to activate gene transcription upon LXR ligand treatment (see Figure 2 and data not shown). As described above, we do not expect these mutations to disrupt ligand binding or overall protein structure. LXR sumoylation and consequent ligand dependent transrepression, therefore, should be maintained. It is possible, however, that mutations to helix 12 or the zinc fingers could influence LXR sumoylation, thereby also disrupting LXR transrepression. Based on the finding that the activities of the glucocorticoid receptor could be dissociated by single point mutations[740], we believe that we can dissociate LXR transactivation and transrepression. If our mutants do not behave as expected we will carry out random mutagenesis of the LXR receptors and use our *in vitro* assays which can be run in high throughput form to identify mutants that dissociate the two activities.

1.2 Characterization of pathway selective LXR mutants in LXR$\alpha^{-/-}\beta^{-/-}$ macrophages

EXPERIMENTAL DESIGN:

Viral Production: Pathway selective LXR mutants identified in AIM 1 will be cloned into lentiviral vectors that co-express GFP. Expression of the mutant LXRs will be under the control of the EF1alpha promoter which expresses well in hematopoietic cells. Empty vector and a vector expressing WT LXR will serve as negative and positive controls, respectively.

BMDM Infection: Bone marrow will be isolated from the femurs of LXR$\alpha^{-/-}\beta^{-/-}$ (DKO) mice and differentiated into macrophages in tissue culture using Monocyte Colony Stimulating Factor conditioned media (M-CSF). Differentiated macrophages will be infected with lentiviral constructs harboring LXR mutants and proper functional analysis will be undertaken. Namely, mRNA and protein levels of LXR mutants will be measured to insure that infected cells have similar expression levels. Additionally, infected macrophages will be examined for their ability

to activate LXR target genes expression in response to ligand treatment and for their ability to repress inflammatory signaling in response to LXR agonist treatment and LPS stimulation. DKO BMDM infected with empty vector alone and WT LXR will serve as negative and positive controls, respectively. All data will be analyzed by 1-way ANOVA followed by Dunnett's post-test.

EXPECTED RESULT: We anticipate that the transactivation and transrepression activity of LXR mutants observed *in vitro* will be recapitulated in primary DKO macrophages infected with lentiviral vectors; thereby identifying LXR mutants that have pathway selective activity *in vivo*.

POTENTIAL PROBLEMS, ALTERNATIVE APPROACHES AND FUTURE DIRECTION:

An alternative approach that we will pursue in parallel is to generate LXR pathway selective transgenic mice in the DKO background. In brief, we will provide the University of Virginia Gene Targeting and Transgenic Facility (GTTF) with DNA constructs containing either LXR mutant or WT LXR (positive control). Following generation of the transgenic lines proper functional analysis will be undertaken as outlined above. The GTTF has prior success generating transgenics in knockout mice on the same background as our DKO mice (C57bl6/J); therefore, we believe this is a reasonable approach.

AIM 2: Determine the relative contribution of LXR regulated pathways to atherosclerosis

RATIONALE: Our data suggests that synthetic LXR agonists do not require RCT[529]; however, LXR expression in immune cells is necessary for their anti-atherogenic activity[549]. Therefore, we hypothesize that the anti-inflammatory properties of LXRs in macrophages are responsible for the anti-atherogenic activity of LXR agonists. To test this hypothesis, we will infect DKO hematopoietic stem cells with pathway selective LXR mutants and then transplant infected cells into irradiated *Ldlr*$^{-/-}$ recipient mice in an atherosclerosis study.

EXPERIMENTAL DESIGN:

Hematopoietic stem cell (HSC) isolation and viral infection: Bone marrow cells from DKO mice enriched in hematopoietic reconstitution activity based on their capacity to exclude the vital dye Hoechst (termed side population, or "SP" cells) will be isolated using fluorescence-activated cell sorting after Hoechst staining[741]. DKO SP cells will be incubated with lentivirus containing LXR mutants in serum-free media containing polybrene and minimal cytokine stimulation (Stem Cell Factor, SCF, and Thrombopoietin, TPO) for 24 hrs. Infected cells will be isolated by FACS sorting for GFP expression.

Bone marrow transplantation and atherosclerosis study: Infected DKO SP cells expressing LXR mutants will be used to reconstitute irradiated *Ldlr*$^{-/-}$ mice in an atherosclerosis study following our published procedures[529, 549]. Our proposed atherosclerosis study will require a total of 90 *Ldlr*$^{-/-}$ recipient mice and 30 DKO mice to serve as bone marrow donors. There will be 15 animals per group as outlined in Table 2. DKO SP cells will be isolated and infected with lentiviral vectors containing mutant LXRs as described above. Four hours after lethal irradiation *Ldlr*$^{-/-}$ recipient mice will be injected via tail vein with ~500 transduced SP cells. This number of SP cells can efficiently reconstitute the hematopoietic system with ~90% of cells expressing the transgene and the transgene expression was shown to last for at least 8 months[742]. Following a recovery period of 4 weeks, *Ldlr*$^{-/-}$ recipients will be bled and LXR mRNA levels will be quantified by RT-PCR of RNA isolated from whole blood. Following confirmation of LXR expression, animals will be switched from standard chow diet to a western diet (21% fat, 0.15% cholesterol) containing vehicle or 0.01% T0901317 and fed *ad libitum*. Mice will be maintained on this diet for 10 weeks, and every 2 weeks animals will be bled to measure plasma lipid levels and mRNA levels of LXR target genes in RNA isolated from whole blood. Inflammation will be

measured using Elisa assays to quantify the amount of TNFα and IL-1β in the plasma. At the conclusion of the study atherosclerosis will be quantitated using our published procedures[529, 549]. Agonist treated mice reconstituted with pathway selective LXRs will be compared to agonist treated mice reconstituted with WT LXR to determine the contribution of LXR transactivation and LXR transrepression to the anti-atherogenic activity of agonist. All data will be analyzed by one way ANOVA followed by Dunett's post-test.

EXPECTED RESULT: We expect LXR agonist treatment will reduce atherosclerosis in *Ldlr*$^{-/-}$ mice receiving wildtype LXR bone marrow, as we have previously shown[549]. Because our recent data[529] indicates that LXR agonist reduces atherosclerosis in the absence of increased RCT, we anticipate that treatment with LXR agonist will reduce atherosclerosis in *Ldlr*$^{-/-}$ animals reconstituted with LXR transrepression selective marrow. On the other hand we expect agonist will have little to no effect in *Ldlr*$^{-/-}$ animals reconstituted with LXR transactivation selective marrow.

POTENTIAL PROBLEMS, ALTERNATIVE APPROACHES AND FUTURE DIRECTIONS

If we cannot achieve sufficient levels of hematopoietic reconstitution using lentiviral infection of DKO we will proceed with the atherosclerosis study once the transgenic animals are in hand. It is possible that transactivation by LXR is in fact required for the anti-atherogenic activity of LXR agonists. LXR transactivates several other pathways that have been proposed to have anti-atherogenic activity in the macrophage, such as macrophage egression[556] and the repression of endoplasmic reticulum stress[695]. Furthermore, as discussed in the preliminary data section, the failure to observe agonist-dependent increases in ^{3}H cholesterol in the plasma of LivKO mice during the *in vivo* RCT experiment (Figure 3.8A) could result from impairments to the RCT pathway that occur after macrophage efflux. Thus, if our study demonstrates that LXR transactivation and not transrepression is required for the anti-atherogenic activity of agonist we will begin to probe these additional LXR regulated pathways. Nevertheless, having the LXR pathway selective transgenic animals will allow us and other to probe the tissue-specific contribution of LXR selective pathways to atherosclerosis as well as other inflammatory and metabolic disorders.

ADDENDUM

In the time since this project proposal was written the Glass lab[614] in collaboration with Olefsky et al. put forth an alternative mechanism to describe the LXR agonist dependent repression of NFκB pro-inflammatory gene expression. Glass and collaborators report in this study that the macrophage-specific deletion of NCoR paradoxically results in an anti-inflammatory phenotype in obese mice. This effect was attributed to the derepression of LXRs resulting in increased expression of genes directing the biosynthesis of ω3 fatty acids. The increased ω3 fatty acid levels were found to inhibit NFκB dependent inflammatory signaling by uncoupling NFκB binding and the enhancer/promoter histone acetylation required for gene activation (Figure 1.6, adopted from Li et al.[614])

It is possible that LXR agonist-dependent transrepression represents an acute mechanism by which LXRs repress NFκB pro-inflammatory networks, whereas NFκB repression by LXR stimulated increases in ω3 fatty acid levels is more of a delayed long-acting response. Both LXR mediated pathways could contribute to the anti-inflammatory activity of LXR agonists in macrophages. In light of these recent findings, there is a possibility that the LXR transrepression defective mutants described above still maintain LXR-agonist dependent anti-inflammatory activity through this novel ω3 mediated NFκB

repression pathway. **To address this possibility I present the following alternative approach:**

Chip-Seq analysis of NCoR deficient macrophages revealed significantly lower levels of TLR4 dependent H3K4me2 deposition at hyporesponsive genes, a pattern that was observed for all NFkB regulated genes reduced in response to LXR-stimulated ω3 levels. H3K4me2 is a histone post-translational modification enriched in cis-regulatory regions, particularly promoters, of transcriptionally active genes[743] . Post-translational modifications of histone tails, especially acetylation and methylation on lysine residues, play a pivotal role in regulating gene expression by controlling the accessibility of chromatin to key regulatory factors. Methylation of H3K4 is associate with open chromatin and transcriptional activation[744]. For lysine residues that are subject to both acetylation and methylation, acetylation can block subsequent methylation, and vice versa, as a result of mutual exclusivity. Recent evidence, moreover, suggests a link between H3 hyperacetylation and increased H3K4 methylation. Indeed, treatment *in vitro* and *in vivo* with various inhibitors of histone deacetylase (HDAC) has been reported to increase levels of H3K4me2. Histone methylation is a reversible process that is regulated by a dynamic balance between histone methyltransferase and histone demethylase activities[745] and HDAC inhibitors increase H3K4me2 levels by repressing demethylase activity. Thus, another way to address the role of LXR anti-inflammatory activity could be through the use of HDAC inhibitors. If any LXR transrepression defective mutants are found to maintain anti-inflammatory effects in the proposed athero study, it will be presumably through the ability of these mutants to still increase ω3 fatty acid synthesis. The concurrent administration of HDAC inhibitors then could increase H3K4me2 levels thereby eliminating the protective effects of LXR-stimulated ω3 levels. Additionally, inhibitors of fatty acid synthesis could similarly be employed to eliminate LXR-stimulated ω3 levels. Thus, if *Ldlr*$^{-/-}$ mice transplanted with LXR transrepression defective bone marrow are found to maintain LXR agonist dependent anti-inflammatory activity an additional arm of the study would be to treat a cohort of these animals (and controls) with either an HDAC inhibitor or an inhibitor of fatty acid synthesis. HDAC inhibition may have many effects on LXR regulated gene expression; thus if HDAC inhibition is found to reduce LXR agonist-stimulated transactivation, fatty acid synthesis inhibition may be the more appropriate approach.

LXR subtype	Mutant	Expectation	Trans-Repress	Trans-activate
β	K410R, K448R	Disrupt sumoylation	-	+
α	K328R, K434R	Disrupt sumoylation	-	+
β	S427D	Disrupt interaction with transrepression complex	-	+
β	L452A	Destabilize helix 12; disrupt interaction with co-activator	+	-
α	L438A	Destabilize helix 12; disrupt interaction with co-activator	+	-
β	C104A, C107A	Disrupt DNA binding	+	-
α	C115A, C118A	Disrupt DNA binding	+	-

Table 1 ***LXR Mutants***

Group	Marrow	Drug Tx
1	TA+	Vehicle
2	TA+	T0901317
3	TR+	Vehicle
4	TR+	T0901317
5	Wildtype	Vehicle
6	Wildtype	T0901317

Table 2 ***Atherosclerosis study with pathway specific LXR bone marrow.*** *Ldlr*$^{-/-}$ recipient mice (N=15/group) will be reconstituted with LXR transactivation selective (TA+), LXR transrepression selective (TR+) or WT LXR marrow.

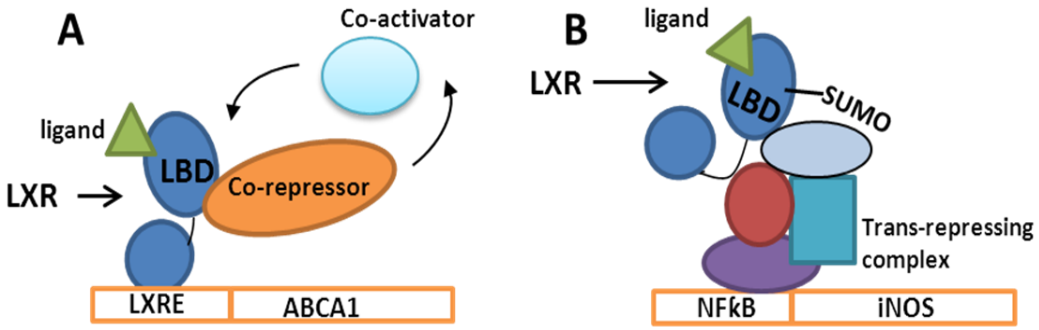

Figure 1 ***LXR activity*** Ligand-dependent transactivation (**A**) and transrepression (**B**). See text for details.

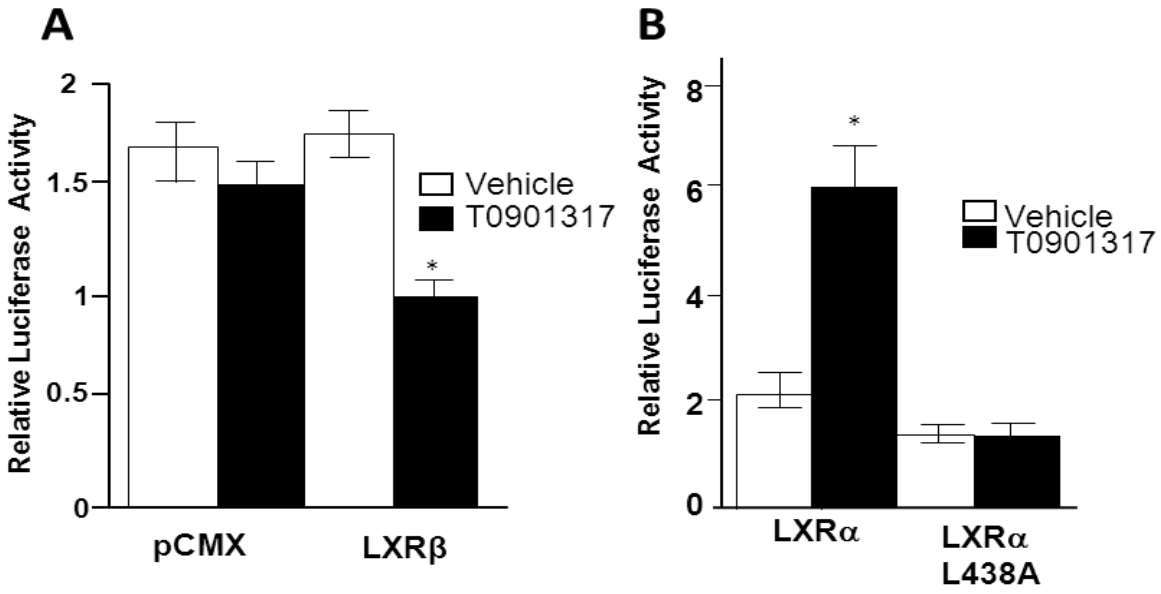

Figure 2 ***In vitro LXR activity*****.**

Figure 6.2 ***In vitro LXR activity*****. A**) LXR and a luciferase reporter gene containing 3-NFκB binding sites from the human Il-8 promoter were co-transfected into Hela cells with a B-galactosidase plasmid. Following 24 hr incubation in the absence or presence of 1uM T0901317, cells were stimulated with TNFα (1ng/ml) for 6 hrs. Cells were lysed and luciferase activity normalized to B-gal. **B**) LXR and a luciferase reporter gene containing 3 upstream LXREs were co-transfected into HEK293T cells with a B-galactosidase plasmid and cultured in the absence or presence of 1uM T0901317 for 24 hrs. Cells were lysed and luciferase activity normalized to B-gal. Luciferase activity was normalized to B-Gal. * ≤ 0.05% compared to vehicle treated. Data was analyzed by 1-way ANOVA followed by Dunnett's post-test.

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Publications resulting from this work

Journal articles

Breevoort SR, Angdisen J, Schulman IG. Macrophage Independent Regulation of Reverse Cholesterol Transport by Liver X Receptors. **2014**. ATVB. Submitted

Zhang Y*, **Breevoort SR***, Angdisen J, Fu M, Schmidt DR, Holmstrom SR, Kliewer SA, Mangesdorf DJ, Schulman IG. Liver LXRα is crucial for whole body cholesterol homeostasis and reverse cholesterol transport in mice. **2012**. *J Clinic invest.* 122(5):1688-99. PMCID: PMC3336978
** co-first author*

Conference Abstracts

Sarah Breevoort, Jerry Angdisen and Ira G. Schulman **(January 2014)** Macrophage Independent Regulation of Reverse Cholesterol Transport by Liver X Receptors. Poster presentation at Keystone Symposia on Nuclear Hormone Receptors, Taos, NM.

Sarah Breevoort, Jerry Angdisen and Ira G. Schulman **(September 2013)** The Liver X Receptors and Reverse Cholesterol Transport. Oral presentation at S.

Sarah Breevoort, Jerry Angdisen and Ira G. Schulman. Liver LXRα plays a critical role in regulating LXR-dependent Reverse Cholesterol Transport.

- Poster presentation, Deuel Lipid Meeting, Napa, CA – **March 2013**
- Poster presentation, Southeast Regional Lipid Meeting, Pine Mountain, GA – **September 2013**

Sarah Breevoort, Jerry Angdisen,Yuan Zhang, Steven A. Kliewer, David J. Mangelsdorf, and Ira G. Schulman. LXRα regulates liver-dependent and - independent pathways to limit atherosclerosis

- Poster presentation, Keystone Symposia, Molecular Basis of Vascular Inflammation and Atherosclerosis, Big Sky, MT – **March 2012**
- Poster presentation, American Physician Scientist Association (APSA) conference, Chicago, IL – **April 2012**
- Poster presentation, Robert Berne Cardiovascular Center Symposium, Charlottesville, VA – **May 2012**