

Genetic Deletion of Low Density Lipoprotein Receptor Impairs Sterol-induced Mouse Macrophage ABCA1 Expression

A NEW SREBP1-DEPENDENT MECHANISM*

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Low density lipoprotein receptor (LDLR) mutations cause familial hypercholesterolemia and early atherosclerosis. ABCA1 facilitates free cholesterol efflux from peripheral tissues. We investigated the effects of LDLR deletion (LDLR^{-/-}) on ABCA1 expression. LDLR^{-/-} macrophages had reduced basal levels of ABCA1, ABCG1, and cholesterol efflux. A high fat diet increased cholesterol in LDLR^{-/-} macrophages but not wild type cells. A liver X receptor (LXR) agonist induced expression of ABCA1, ABCG1, and cholesterol efflux in both LDLR^{-/-} and wild type macrophages, whereas expression of LXR α or LXR β was similar. Interestingly, oxidized LDL induced more ABCA1 in wild type macrophages than LDLR^{-/-} cells. LDL induced ABCA1 expression in wild type cells but inhibited it in LDLR^{-/-} macrophages in a concentration-dependent manner. However, lipoproteins regulated ABCG1 expression similarly in LDLR^{-/-} and wild type macrophages. Cholesterol or oxysterols induced ABCA1 expression in wild type macrophages but had little or inhibitory effects on ABCA1 expression in LDLR^{-/-} macrophages. Active sterol regulatory element-binding protein 1a (SREBP1a) inhibited ABCA1 promoter activity in an LXRE-dependent manner and decreased both macrophage ABCA1 expression and cholesterol efflux. Expression of ABCA1 in animal tissues was inversely correlated to active SREBP1. Oxysterols inactivated SREBP1 in wild type macrophages but not in LDLR^{-/-} cells. Oxysterol synergized with non-steroid LXR ligand induced ABCA1 expression in wild type macrophages but blocked induction in LDLR^{-/-} cells. Taken together, our studies suggest that LDLR is critical in the regulation of cholesterol efflux and ABCA1 expression in macrophage. Lack of the LDLR impairs sterol-induced macrophage ABCA1 expression by a sterol regulatory element-binding protein 1-dependent mechanism that can result in reduced cholesterol efflux and lipid accumulation in macrophages under hypercholesterolemic conditions.

There is a high degree of correlation of plasma cholesterol levels with the incidence of coronary heart disease. It has been

shown that plasma cholesterol homeostasis is dependent on ingestion, synthesis, and metabolism of cholesterol (1). Similarly, it has been demonstrated that both the low density lipoprotein receptor (LDLR)³ and the ATP binding cassette transporter A1 (ABCA1) play key regulatory roles in cellular cholesterol metabolism (2, 3). The LDLR is a cell surface-glycoprotein that endocytoses cholesterol bound to LDL, and it is responsible for the clearance of about 70% of plasma LDL cholesterol in human liver (2, 4). The LDLR also binds β -VLDL (very low density lipoprotein) and certain intermediate density lipoprotein and HDL (5).

Human LDLR mutations cause familial hypercholesterolemia (FH), a common autosomal dominant disorder that affects approximately 1 in 500 individuals in the heterozygous form with about a 2-fold elevation in plasma LDL cholesterol (6, 7). Without treatment, excess LDL cholesterol can deposit in tendons and arteries. It eventually leads to the formation of tendon xanthomas and atherosclerotic plaques (8). Clinically overt coronary heart disease caused by heterozygous FH is observed at an age of 45–48 years in males and of 55–58 years in females (9). The homozygous FH patients are rare (~1:10⁶). However, when it occurs, one sees a 4–5-fold elevation in plasma LDL cholesterol with marked cutaneous tuberous xanthomas. Often, there are frequent myocardial infarctions at the age of 20–30 years (9). In mice, genetic deletion of the LDLR (LDLR knock-out, LDLR^{-/-}) will cause a moderate increase in plasma LDL cholesterol when these animals are fed a normal chow. However, a severe elevation of LDL plasma cholesterol is associated with aortic lesions when mice are fed a Western diet (10). Thus, LDLR^{-/-} mice are used as a model for the study of the pathogenesis of atherosclerosis and FH (11).

ABCA1 and ABCG1 belong to the large family of ATP-binding cassette transporters. By using the energy from the hydrolysis of ATP, ABCA1 and ABCG1 facilitate cholesterol efflux from macrophages and other cell types to apoAI and/or HDL (12). ABCA1 mutations cause Tangier disease, characterized by very low levels of HDL cholesterol, poorly lipidated and rapidly catabolized apoAI, cholesteryl ester accumulation in peripheral tissues, and a high risk of development of coronary heart disease (13, 14). In apoE knock-out mice, overexpression of human

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³ The abbreviations used are: LDLR, low density lipoprotein (LDL) receptor; HDL, high density lipoprotein; AcLDL, acetylated LDL; OxLDL, oxidized LDL; ABCA1, ATP binding cassette transporter A1; FH, familial hypercholesterolemia; LXR, liver X receptor; LXRE, LXR response element; SREBP, sterol regulatory element-binding protein; PBS, phosphate-buffered saline.

LDLR Deletion Impairs Macrophage ABCA1 Expression

ABCA1 reduces total cholesterol levels and atherosclerosis, whereas selective suppression of macrophage ABCA1 increases atherosclerosis without affecting total cholesterol levels (15, 16). In contrast, the activation of ABCG1 expression can promote atherosclerotic lesion development whereas inactivation of macrophage ABCG1 expression reduces it in proatherogenic mice (17, 18).

ABCA1 has a half-life of about 1–2 h. Thus, decreased ABCA1 degradation by apoA1 results in increased ABCA1 levels, whereas enhanced ABCA1 degradation by unsaturated fatty acids decreases ABCA1 levels in macrophages (19, 20). Loading of wild type macrophages with lipoproteins or sterols can increase ABCA1 expression and cholesterol efflux, thus potentially preventing uncontrolled lipid accumulation and formation of lipid-laden macrophage/foam cells (21, 22).

Several transcription factors have been defined to play a critical role in the regulation of ABCA1 expression and cholesterol efflux (23). LXR α/β (ligand-activated transcription factors) can increase ABCA1/ABCG1 expression (24, 25), whereas administration of synthetic LXR ligands to proatherogenic mice will result in significantly reducing atherosclerosis (26–28). However, clinical use of LXR ligands has been limited by very low density lipoprotein-triglycerides accumulation in the liver (29).

Sterol regulatory element-binding proteins 1 and 2 (SREBP1/2), the nuclei membrane-bound transcription factors, regulate expression of genes involved in cholesterol synthesis, endocytosis of LDL, synthesis of fatty acids, and glucose metabolism. Activity of SREBPs is feedback inhibited by cellular sterol levels (30, 31). SREBP2 has been reported to inhibit ABCA1 expression in human endothelial cells by binding the E-box in the ABCA1 promoter (32). However, a separate report suggests that binding of SREBP2 to the E-box does not alter the E-box activity (33). Recently, it has been shown that SREBP2 is a positive regulator of ABCA1 expression in transfected CHO-7 cells (34).

Expression of SREBP1 and SREBP2 is cell type-dependent. SREBP1c and SREBP2 are predominantly expressed by the liver (35). In contrast, SREBP1a with 29-additional acidic-rich amino acids at the N terminus and more potent transcriptional activity is widely expressed by most tissues (35). Because of the existence of the sterol regulatory element in promoters of SREBP1 and SREBP2, they may cross-regulate each other (36, 37). The role of SREBP1 or SREBP2 on expression of ABCA1, particularly in macrophages, is unknown.

Although the role of the LDLR in the mediation of uptake and clearance of LDL cholesterol by hepatocytes has been well defined, it is unclear if the LDLR plays a role in macrophage ABCA1 expression and cholesterol efflux. Cellular endocytosis of LDL cholesterol is reduced in the absence of the LDLR. However, deletion of LDLR leads to atherosclerosis in hypercholesterolemic mice. In addition to the contribution by scavenger receptor-mediated uptake of modified LDL, we hypothesized that lesion development is also due to the defect in macrophage cholesterol efflux. In this study we tested the hypothesis that lack of LDLR will impair sterol-induced macrophage ABCA1 expression. We demonstrate for the first time that the LDLR plays an important role in regulating macrophage cholesterol efflux. We show that LDLR deletion reduces basal levels of

ABCA1, ABCG1, and cholesterol efflux from macrophages and significantly reduces sterol-induced ABCA1 expression in macrophages. Furthermore, we show that these effects are mediated by an abnormal response of SREBP1 to sterols.

EXPERIMENTAL PROCEDURES

Reagents—All chemicals were purchased from Sigma-Aldrich. A high fat diet (21% fat and 0.2% cholesterol) was purchased from Harlan Teklad (Madison, WI). [^3H]Cholesterol was purchased from PerkinElmer Life Sciences. Total and free cholesterol assay kits were obtained from Wako Chemicals USA, Inc. (Richmond, VA). Rabbit anti-mouse ABCA1 and ABCG1 polyclonal antibodies were purchased from Novus Biologicals, Inc. (Littleton, CO). Rabbit anti-mouse SREBP-2 specific polyclonal antibody was a kind gift from Dr. Joseph Goldstein of The University of Texas Southwestern Medical Center. All other antibodies were polyclonal, and they were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Cell Culture—Adult mice (both wild type (C57BL) and LDLR knock-out with the same background as C57BL, 10–12 weeks) were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in fully accredited facilities (Assessment and Accreditation of Laboratory Animal Care) at Weill Cornell Medical College. Mice were injected intraperitoneal (3 ml/mouse) with 4% autoclaved thioglycolate solution and kept for 5 days with free access to drinking water and chow. Peritoneal macrophages were isolated by lavage of the abdomen with PBS (2 \times 8 ml) after sacrifice. They were cultured (density at 300 \times 10³ cells/cm²) in complete RPMI medium containing 10% fetal calf serum, 50 $\mu\text{g}/\text{ml}$ penicillin and streptomycin, and 2 mM glutamine. After 3 h culture, floating cells (most are red blood cells) were removed by washing with PBS. Adherent cells were maintained in complete medium. Peritoneal macrophages were isolated from mice fed a normal chow except indicated.

Determination of Free and Total Cholesterol Levels in Peritoneal Macrophages—Wild type or LDLR^{-/-} mice at 10 weeks of age were fed a normal chow or a high fat diet for 4 weeks. Peritoneal macrophages from each mouse were individually collected. After extraction, macrophage cellular lipids were used to determine free and total cholesterol levels as described (38).

Isolation of LDL and HDL and Preparation of ApoAI, Acetylated LDL (AcLDL), and Oxidized LDL (OxLDL)—LDL (1.019–1.063 g/ml) and HDL (1.063–1.21 g/ml) were isolated from normal human plasma by sequential ultracentrifugation, dialyzed against PBS containing 0.3 mM EDTA, sterilized by filtration through a 0.22- μm filter, and stored under N₂ gas at 4 °C. Protein content was determined by the Lowry method. ApoAI was isolated from purified HDL by removal of lipids with an extraction solution of acetone and ethanol (1:1) (39). Purity of apoAI was determined by SDS-PAGE. AcLDL and OxLDL were prepared as described elsewhere (40).

Determination of Free Cholesterol Efflux from Macrophages—Macrophages in 12-well plates were labeled in macrophage serum-free medium (Invitrogen, 1.5 ml/well) containing 50 $\mu\text{g}/\text{ml}$ AcLDL and 150 nCi/ml [^3H]cholesterol for 24 h. After removal of labeling medium, cells were washed twice with PBS and incubated for 1 h in serum-free medium, then switched to

TABLE 1

Sequences of primers for ABCA1 promoters

Promoter	Template DNA	5'-primer	3'-primer
A ^a (-179 to +227)	Mouse genomic DNA	TAGCCTCGAGGTCGCCGGTTTAAGGGGCG	TGCCAAGCTTCCTCTTACCTGTTTTCCACTTTG
B ^b (E-box deleted)	Promoter A	GGCCATGTCTCCTTTTCGC	GCAGAAAGGAGACATGGCC
C ^b (E-box mutated)	Promoter A	GGCGGGCCATGTCTCCTACTAGACTTTCTGC	GCAGAAAGTCTAGTGAGACATGGCCCGCC
D (-113 to +227)	Mouse genomic DNA	TAGCCTCGAGCAGAGCCGGGAACGGGGCG	Same as promoter A
E (-38 to +227)	Mouse genomic DNA	TAGCCTCGAGCAGTAATTCCGAGGGCGAG	Same as promoter A
F (-179 to -94)	Mouse genomic DNA	Same as promoter A	TGCCAAGCTTCGCCCTTCCCGGCC

^a After sequence was confirmed, the PCR product was digested by XhoI and HindIII and then subcloned into pGL4 luciferase reporter vector (Promega) followed by transfection and amplification.

^b The hexanucleotides of the E-box (CACGTG) was deleted in primers for promoter B or replaced by underlined hexanucleotides in primers for promoter C. PCR was performed by using QuikChange® II site-directed mutagenesis kit (Stratagene, La Jolla, CA) and DNA of promoter A.

serum-free medium or medium containing purified apoAI or HDL (1.5 ml/well). After 5 h of incubation at 37 °C, medium from each well was collected for the determination of radioactivity in the supernatants. Remaining cells in the wells and Eppendorf tubes were lysed by addition of 0.2 N NaOH, and the combined lysate was determined for protein content which was used to normalize cholesterol efflux (dpm/μg of protein).

Northern Blot Analysis of ABCA1, ABCG1, LXRα, and LXRβ mRNA Expression—Total cellular RNA was extracted from cells in 60-mm dishes and used to determine expression of ABCA1, ABCG1, LXRα, and LXRβ mRNA by Northern blot as described (40). The probes for mouse ABCA1, ABCG1, LXRα, and LXRβ were generated by reverse transcription-PCR using the following primers: ABCA1, forward (F)-TGGACATCCTGAAGCCAG and backward (B)-TTCTTCCCACATGCCCT; ABCG1, F-GCTGGGAAGTCCACACTC, B-GATACGGCAGGAGATTGG; LXRα, F-GGCAACACTTGCATCCTC, B-CTGTAGGAAGCCAGGGAG; LXRβ, F-GAGCCAGAGGATGAGCCT and B-GACTTTGGGCTGGTCCGGAG.

Western Blot Analysis of ABCA1, ABCG1, SREBP1, and SREBP2 Protein Expression—Total cellular proteins were extracted as described (40) and separated on a 7% (for ABCA1) or 12% (for ABCG1, SREBP1, and SREBP2) SDS-PAGE followed by Western blot analysis.

To determine the nuclear form of SREBP1, nuclear proteins were extracted as follows. Cells were suspended in cold buffer A (20 mM Hepes, pH 7.9, 10 mM NaCl, 3 mM MgCl₂, 0.1% Nonidet P-40, 10% glycerol, 0.2 mM EDTA, 50 μg/ml each of aprotinin, and leupeptin) and incubated on ice for 20 min followed by centrifugation for 5 min with a microcentrifuge at 3000 rpm and 4 °C. Pellets of nuclei were washed once with buffer A and then buffer B (20 mM Hepes, pH 7.9, 20% glycerol, 0.2 mM EDTA, 50 μg/ml each of aprotinin and leupeptin). Nuclear pellets were resuspended in buffer C (20 mM Hepes, pH 7.9, 0.4 M NaCl, 20% glycerol, 0.2 mM EDTA, 50 μg/ml each of aprotinin and leupeptin) and incubated on ice for 1 h with vortexing several times. The suspension was centrifuged for 30 min at 14,000 rpm and 4 °C. The supernatants were collected, separated into aliquots, and stored at -20 °C until assay.

Plasmid DNA Preparation and Transfection—cDNA encoding nuclear form of mouse SREBP1a (N-terminal 1–460 amino acids) was generated by PCR using the clone purchased from Invitrogen (cDNA clone MGC:66503 IMAGE:6824948) as a template DNA and the primers F-ACTCAGATCTCGATGACGAGCTGGCCTTCG and B-CCGCGGTACCCTAGACTGGCTATCCTCAAAGG. After the sequence was confirmed, the PCR product was digested with BglII and KpnI and

then subcloned into an expression vector, pEGFP-C2 (pEGFP-nSREBP1a).

Mouse ABCA1 promoters were constructed by PCR using the mouse genomic DNA and primers as shown in Table 1. For promoter activity assay, about 95% of the confluent 293 cells in 24-well plates were co-transfected with the DNA for the ABCA1 promoter, nSREBP1a, and *Renilla* (for internal normalization) by using Lipofectamine™ 2000 (Invitrogen). After 24 h of transfection, cells were lysed, and the cellular lysate was used to determine the activity of firefly and *Renilla* luciferases by using the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI).

Data Analysis—All experiments were repeated at least three times, and representative results are presented. Data were generated from the cholesterol assays, and the cholesterol efflux studies or the activity assays involving the ABCA1 promoters were analyzed by paired *t* test.

RESULTS

High Fat Diet Induces Cholesterol Accumulation in LDLR^{-/-} Macrophages—To determine whether LDLR deletion alters macrophage cholesterol levels, both wild type and LDLR^{-/-} mice were fed a normal chow or a high fat diet. After 4 weeks of feeding, mice were used to collect samples of plasma as well as peritoneal macrophages. The high fat diet increased total and free cholesterol levels in the plasma of both types of mice but with a greater effect in the LDLR^{-/-} mice (Table 2). For instance, both total and free cholesterol levels in the plasma of the wild type mice were increased ~2-fold in those fed a high fat diet. In contrast, the high fat diet resulted in about a 4- and 5-fold increase in total and free plasma cholesterol levels in LDLR^{-/-} mice.

Interestingly, the total or free cellular cholesterol levels in macrophages were similar between wild type and LDLR^{-/-} mice when they were fed a normal chow, albeit the plasma total and free cholesterol level in LDLR^{-/-} mice were about 5-fold higher than wild type mice. Administration of a high fat diet increased plasma cholesterol levels in wild type mice, but it did not affect the cellular total or free cellular cholesterol levels in these macrophages (Table 2). In contrast, a high fat diet increased total cholesterol levels ~2-fold in LDLR^{-/-} macrophages (Table 2). The increase was due to the accumulation of cholesteryl esters since the free cholesterol levels were not changed.

LDLR Deletion Does Not Change Expression of Scavenger Receptors but Reduces Efflux of Free Cholesterol and Expression

LDLR Deletion Impairs Macrophage ABCA1 Expression

TABLE 2

A high fat diet induces plasma and macrophage cholesterol accumulation in LDLR^{-/-} mice

Blood and peritoneal macrophages were collected from individual mice after 4 weeks of normal chow or a high-fat diet feeding. Total and free cholesterol were determined by using an assay kit from Wako Chemicals.

Genotype	Serum				Macrophage			
	Normal chow		High fat diet		Normal chow		High fat diet	
	Total	Free	Total	Free	Total	Free	Total	Free
	mg/dl				μg/mg of protein			
Wild type	51 ± 7	13 ± 1	109 ± 10 ^a	27 ± 2 ^a	50 ± 7	47 ± 5	48 ± 5	46 ± 3
LDLR ^{-/-}	235 ± 23 ^b	75 ± 8 ^b	1092 ± 108 ^{b,c}	366 ± 42 ^{b,c}	48 ± 6	45 ± 4	114 ± 8 ^d	41 ± 2

^a High fat diet vs. normal chow in wild type.

^b LDL^{-/-} vs. wild type in serum.

^c High fat diet vs. normal chow in LDL^{-/-}.

^d LDL^{-/-} vs. wild type in macrophages.

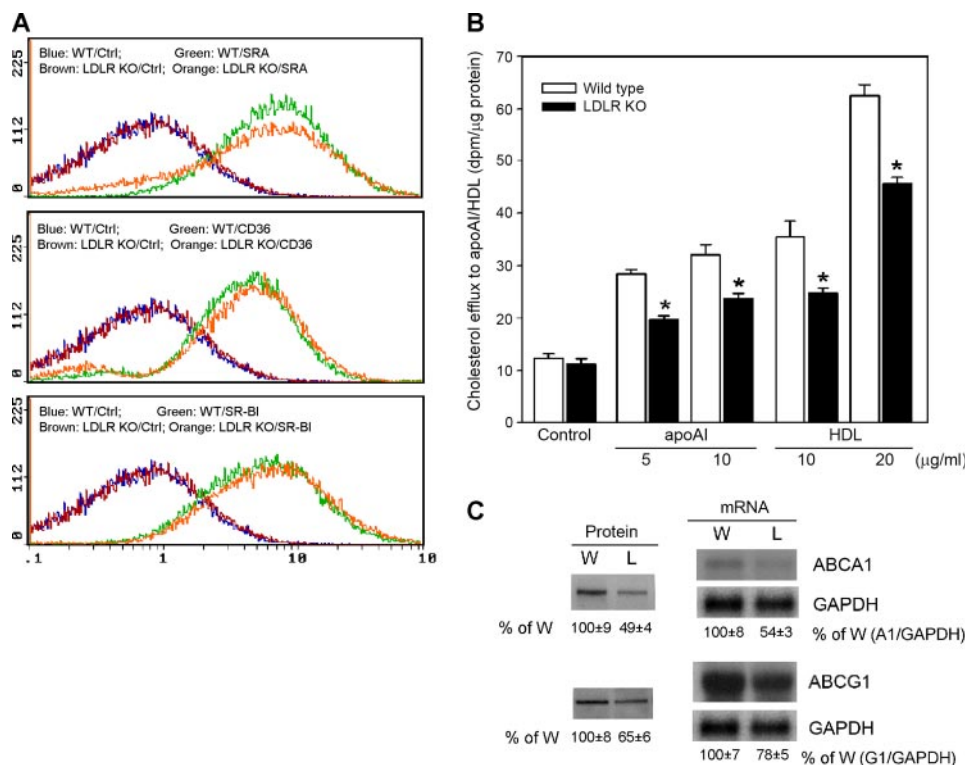


FIGURE 1. LDLR^{-/-} does not alter expression of scavenger receptors but reduces cholesterol efflux and expression of ABCA1 and ABCG1 in macrophages. A, macrophages were isolated from wild type (WT) and LDLR^{-/-} mice and used to determine expression of type A (SRA), type B (CD36), or type BI (SR-BI) scavenger receptor by fluorescence-activated cell sorter as follows. Cells (~1 × 10⁶ cells/sample) were blocked for 30 min at room temperature with PBS containing 5% goat serum. After washing, cells were incubated with rabbit anti-SRA or CD36 or SR-BI antibody (1:100, control cells were added normal rabbit IgG) for 1 h at room temperature. Cells were then incubated with goat anti-rabbit fluorescein isothiocyanate-conjugated IgG (1:50) for 45 min. After washing with PBS, cells were subjected to fluorescence-activated cell sorter. Ctrl, Control; KO, knock out. B, wild type and LDLR^{-/-} macrophages were cultured in 12-well plates and used to conduct free cholesterol efflux. Data were expressed as the mean ± S.D. *, significantly different from the corresponding wild type cells (*p* < 0.05, *n* = 4). C, total cellular proteins and RNA were extracted from wild type and LDLR^{-/-} macrophages. Cellular proteins (40 μg) from each sample were loaded on a 7 or 12% SDS-PAGE to analyze ABCA1 or ABCG1 protein by Western blot. Total cellular RNA (20 μg) was used to determine ABCA1 or ABCG1 mRNA by Northern blot. Densitometric analysis of autoradiograms of Western and Northern blots was completed by using a UMAX (Santa Clara, CA) UC630 flatbed scanner and NIH Image software (*n* = 3). GAPDH, glyceraldehyde-3-phosphate dehydrogenase. W, wild type; L, LDLR^{-/-}.

of ABCA1 and ABCG1 in Macrophages—To study the pathways by which the high fat diet induced cholesterol accumulation in LDLR^{-/-} macrophages, we initially focused on the effects of LDLR deletion on the expression of scavenger receptors. Type A and type B scavenger receptors (SRA and CD36) are major proteins responsible for the binding and internalization of modified LDL in macrophages (41, 42). Data in Fig. 1A

indicated that the expression of SRA or CD36 was not affected by LDLR deletion. Type BI scavenger receptor (SR-BI), a molecule mediating both free cholesterol efflux to HDL from cells and selective uptake of cholesteryl esters by cells (43), was also not changed (Fig. 1A). Thus, the major pathways for uptake of cholesterol remained unaffected in the absence of LDLR.

ABCA1 and ABCG1 facilitate macrophage-free cholesterol efflux to apoAI and/or HDL. Alteration in the expression of ABCA1 and/or ABCG1 in macrophages also impacts on cellular cholesterol homeostasis. To determine whether the cholesterol accumulation in LDLR^{-/-} macrophages was due to a defect in the cholesterol efflux pathway, free cholesterol efflux from macrophages to apoAI or HDL was assessed. Compared with wild type macrophages, cholesterol efflux to either apoAI or HDL from LDLR^{-/-} cells was decreased (Fig. 1B).

To correlate the decreased cholesterol efflux from LDLR^{-/-} macrophages with the expression of ABCA1 and ABCG1, cellular RNA or proteins were extracted from macrophages and used to assess ABCA1 or ABCG1 mRNA and protein by Northern and Western blot. LDLR deletion resulted in a moderate reduction in ABCG1 (~20–30%) and a greater reduction in ABCA1 (~50%) at the mRNA and protein levels (Fig. 1C).

LDLR Deletion Does Not Affect LXR Ligand-induced Macrophage Cholesterol Efflux and ABCA1 or ABCG1 Expression—To determine whether LDLR deletion affects the LXR pathway, wild type and LDLR^{-/-} macrophages were treated with a synthetic nonsteroid LXR ligand, T0901317, followed by assess-

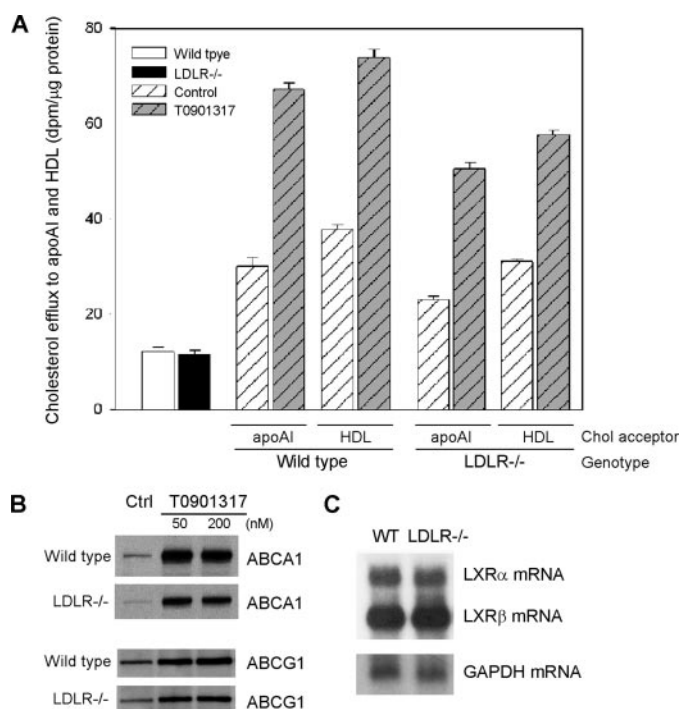


FIGURE 2. LDLR deletion does not interrupt LXR ligand-induced macrophage ABCA1, ABCG1 expression, and cholesterol efflux. A, prelabeled wild type or LDLR^{-/-} macrophages in 12-well plates were treated with 200 nM T0901317 overnight followed by determination of free cholesterol (Chol) efflux to apoA1 (10 μg/ml) or HDL (20 μg/ml). Data were expressed as the mean ± S.D. (n = 4). B, wild type and LDLR^{-/-} macrophages in serum-free medium were treated with T0901317 at the indicated concentrations overnight. After extraction, total cellular proteins (40 μg) were used to determine ABCA1 and ABCG1 protein expression by Western blot. Ctrl, control. C, total cellular RNA (20 μg) from control wild type (WT) or LDLR^{-/-} macrophages was used to determine expression of LXRα and LXRβ mRNA by Northern blot. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

ment of cholesterol efflux and expression of ABCA1 and ABCG1. T0901317 increased cholesterol efflux to apoA1 and HDL from wild type and LDLR^{-/-} macrophages at similar levels (~2-fold, Fig. 2A). Similarly, T0901317 induced ABCA1 expression in both cell types (Fig. 2B). Another target gene of LXR, ABCG1, was also increased to a similar degree by the LXR ligand, suggesting that LXR-mediated macrophage cholesterol efflux and ABCA1/ABCG1 expression was not influenced by LDLR expression. To further confirm this, we determined the expression of LXRα and LXRβ in macrophages, and we observed that levels in wild type and LDLR^{-/-} macrophages were very similar (Fig. 2C).

LDLR Deletion Impairs Sterol-induced Expression of ABCA1, but Not ABCG1, in Macrophages—Loading of macrophages with sterols increases ABCA1 or ABCG1 expression by activating LXR pathway. To determine whether LDLR deletion would impair this mechanism, wild type and LDLR^{-/-} macrophages were treated with lipoproteins, such as LDL, HDL, and modified LDL (AcLDL and OxLDL). Their effects on ABCA1 expression were then assessed. HDL had no effect (Fig. 3A), whereas AcLDL had similar inductive effects (Fig. 3B) on ABCA1 expression in both wild type and LDLR^{-/-} macrophages. OxLDL induced ABCA1 expression in both cell types but with a greater effect on wild type macrophages (Fig. 3A). LDL induced ABCA1 expression in a concentration-dependent

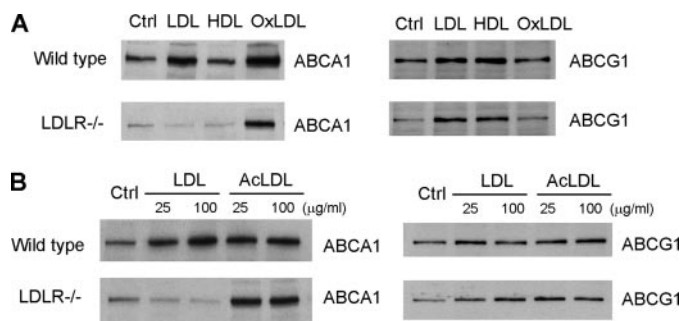


FIGURE 3. Lipoproteins regulate ABCA1 expression differently but ABCG1 expression similarly between wild type and LDLR^{-/-} macrophages. Wild type and LDLR^{-/-} macrophages in serum-free medium were treated overnight as follows: A, LDL, HDL, and OxLDL at 50 μg/ml; B, LDL and AcLDL at the indicated concentrations. Expression of ABCA1 and ABCG1 proteins were then determined by Western blot. Ctrl, control.

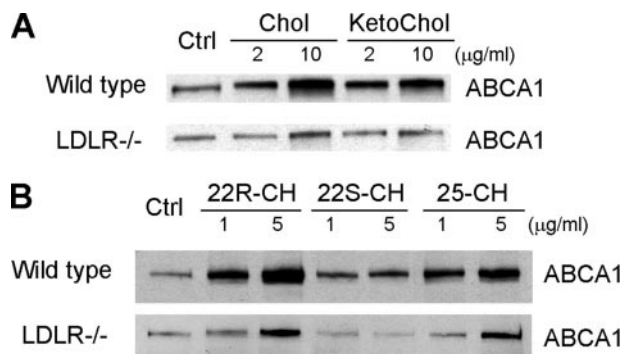


FIGURE 4. Sterols regulate ABCA1 protein expression differently between wild type and LDLR^{-/-} macrophages. Wild type and LDLR^{-/-} macrophages in serum-free medium were treated overnight with cholesterol (Chol) and 7-keto-cholesterol at 2 or 10 μg/ml (A) 22(R)-hydroxycholesterol, 22(S)-hydroxycholesterol, and 25-hydroxycholesterol at 1 or 5 μg/ml (B). Expression of ABCA1 protein was determined by Western blot. Ctrl, control.

manner in wild type macrophages but inhibited it in LDLR^{-/-} macrophages (Fig. 3). In contrast, lipoproteins induced ABCG1 expression in a similar manner between wild type and LDLR^{-/-} macrophages (Fig. 3). These findings suggest that the regulation of macrophage ABCA1 expression is not solely dependent on LXR activity.

To further determine the impact of LDLR deletion on sterol-induced macrophage ABCA1 expression, we first treated cells with cholesterol and 7-keto-cholesterol and then determined any changes in ABCA1 protein. Both cholesterol and 7-keto-cholesterol induced ABCA1 expression in a dose-dependent manner in wild type macrophages. In contrast, their effects on LDLR^{-/-} macrophage ABCA1 expression were markedly diminished (Fig. 4A).

Oxysterols normally activate LXR pathways and inhibit SREBP pathways. To study the effects of oxysterols on ABCA1 expression, macrophages were treated with various oxysterols followed by assessment of ABCA1 protein. In wild type macrophages, all oxysterols induced ABCA1 expression (Fig. 4B). The ability of these oxysterols to induce ABCA1 is as follows: 22(R)-hydroxycholesterol ≥ 25-hydroxycholesterol > 22(S)-hydroxycholesterol. In contrast, the inductive effects of these oxysterols on LDLR^{-/-} macrophage ABCA1 expression was significantly reduced (Fig. 4B).

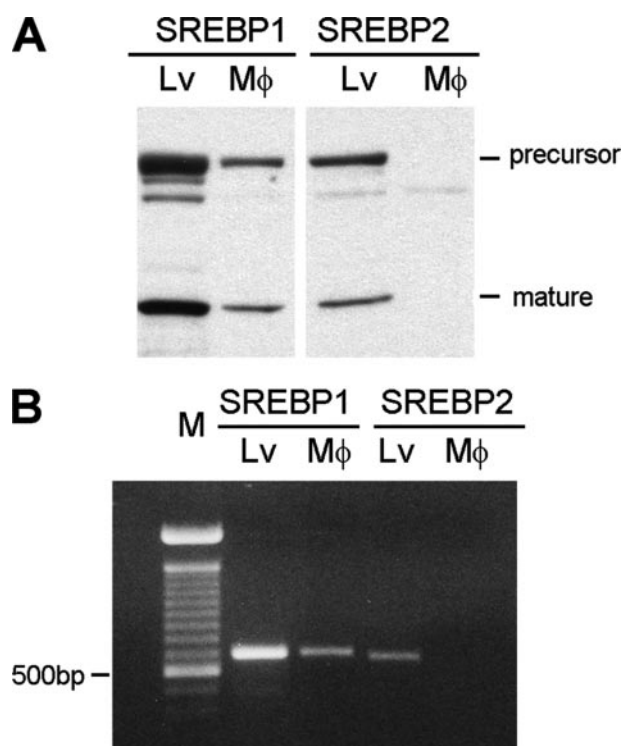


FIGURE 5. Expression of SREBP1 in mouse liver and macrophages. A, expression of SREBP1 or SREBP2 precursor and mature form in whole cellular lysate (40 μ g) of mouse liver (Lv) or macrophages (M ϕ) was determined by Western blot. B, expression of SREBP1 or SREBP2 mRNA was determined by reverse transcription-PCR with following primers: SREBP1, F-GGCAACACTG-CATCCTC and B-CTGTAGGAAGCCAGGGAG (primers cover the common sequence for SREBP1a and SREBP1c); SREBP2, F-CTGGAGGTGGCAGCAATG and B-TTCTGGACTGCAGCCATG.

SREBP1 Is a Negative Regulator of Macrophage ABCA1 Expression—LDLR deletion did not interfere with LXR-induced ABCA1 or ABCG1 expression (Figs. 2 and 3). However, compared with wild type macrophages, the expression of ABCA1 in LDLR^{-/-} macrophages in response to sterols was significantly reduced (Figs. 3 and 4). Because SREBP2 has been reported to inactivate ABCA1 expression in human endothelial cells (32), we determined if SREBP1 or SREBP2 can play a role in the regulation of macrophage ABCA1 expression. Initially, we detected their expression either in precursor or nuclear form in macrophages by Western blot analysis (Fig. 5A). Compared with strong expression in the liver, SREBP1 was moderately expressed, whereas SREBP2 was barely detectable in macrophages. Expression of SREBP1 and lack of expression of SREBP2 by macrophages was confirmed further by reverse transcription-PCR (Fig. 5B).

To determine the effect of SREBP1 on macrophage ABCA1 expression, we constructed several ABCA1 promoters by PCR based on the ABCA1 promoter sequence (Fig. 6A, Table 1). Promoter A, including the E-box and the LXR response element (LXRE), had moderate activity. Deletion of the E-box (CACGTG was deleted, promoter B) or mutation in the E-box (CACGTG was replaced by ACTAGA, promoter C) significantly enhanced ABCA1 promoter activity as previously reported (44). In contrast, deletion of the E-box and the sequence around the E-box (promoter D) did not change ABCA1 promoter activity. However, all of these promoters

were activated by LXR ligand. The absence of the LXRE (promoter E or F) resulted in inactivation of the ABCA1 promoter (less than vector alone) (Fig. 6B).

To test if activation of SREBP1 inhibits ABCA1 promoter activity, we constructed a cDNA encoding SREBP1a nuclear form (N-terminal 1–460 amino acid residues) and subcloned it into the pEGFP-C2 vector (pEGFP-nSREBP1a). 293 cells were co-transfected with pEGFP-nSREBP1a DNA and various ABCA1 promoter DNAs (Fig. 6C). Expression of nSREBP1a was confirmed by Western blot analysis in transfected cells (data not shown). nSREBP1a inhibited promoter A activity in a concentration-dependent manner. Interestingly, nSREBP1a also inhibited activity of the promoter with the E-box deletion (promoters B and D) or the E-box mutation (promoter C). These findings suggest that the inhibitory effect of nSREBP1a on ABCA1 promoter activity is E-box-independent and LXRE-dependent.

To further investigate if nSREBP1a inhibits ABCA1 promoter activity by blocking LXRE, cells were co-transfected with pEGFP-nSREBP1a DNA and promoter D DNA, which lacks the E-box and a surrounded sequence. We then exposed these cells to LXR ligand. LXR ligand induced promoter D activity, but nSREBP1a reduced such induction in a concentration-dependent manner (Fig. 6D).

The effect of nSREBP1a on macrophage ABCA1 expression was next determined by Western blot analysis (Fig. 7A). Increased nSREBP1a expression resulted in reduced ABCA1 protein expression. We further determined the effect of nSREBP1a on macrophage cholesterol efflux. Associated with the increased nSREBP1a was the reduced macrophage cholesterol efflux (Fig. 7B).

Impairment of Sterol-induced ABCA1 Expression Is Due to the LDLR Deletion-mediated Activation of SREBP1—To determine the correlation between expression of ABCA1 and SREBP1, we extracted proteins from mouse tissues and evaluated levels of ABCA1 and SREBP1 proteins. Among the tested tissues, SREBP1 was least expressed by the lung, whereas ABCA1 was expressed mostly by the lung. Expression of SREBP1 by other tissues (e.g. heart, liver, and kidney) was high, but almost no ABCA1 was detected (Fig. 8A).

To test the hypothesis that impairment of sterol-induced LDLR^{-/-} macrophage ABCA1 expression is mediated by SREBP1 activity, we treated cells with oxysterols and determined their effect on SREBP1 activity (Fig. 8B). In wild type macrophages, 22(R)- and 25-hydroxycholesterol significantly reduced nSREBP1 at concentrations of 1 and 5 μ g/ml. 22(S)-hydroxycholesterol also reduced nSREBP1 but with a lesser effect. Surprisingly, in LDLR^{-/-} macrophages, 22(R)- and 25-hydroxycholesterol had little effect on nSREBP1, whereas 22(S)-hydroxycholesterol increased nSREBP1, suggesting that SREBP1 processing was improper in response to sterols in the absence of LDLR (Fig. 8B).

In wild type cells, sterols inhibit nSREBP1. In contrast, sterols can activate nSREBP1 in LDLR^{-/-} macrophages. To test the effects of sterols on nonsteroid LXR ligand-mediated ABCA1 expression, we co-treated macrophages with T0901317 and 22(S)-hydroxycholesterol. Results in Fig. 8C demonstrate that 22(S)-hydroxycholesterol synergizes with T0901317-induced ABCA1 expression in wild type macrophages; the opposite effects occur in LDLR^{-/-} macrophages.

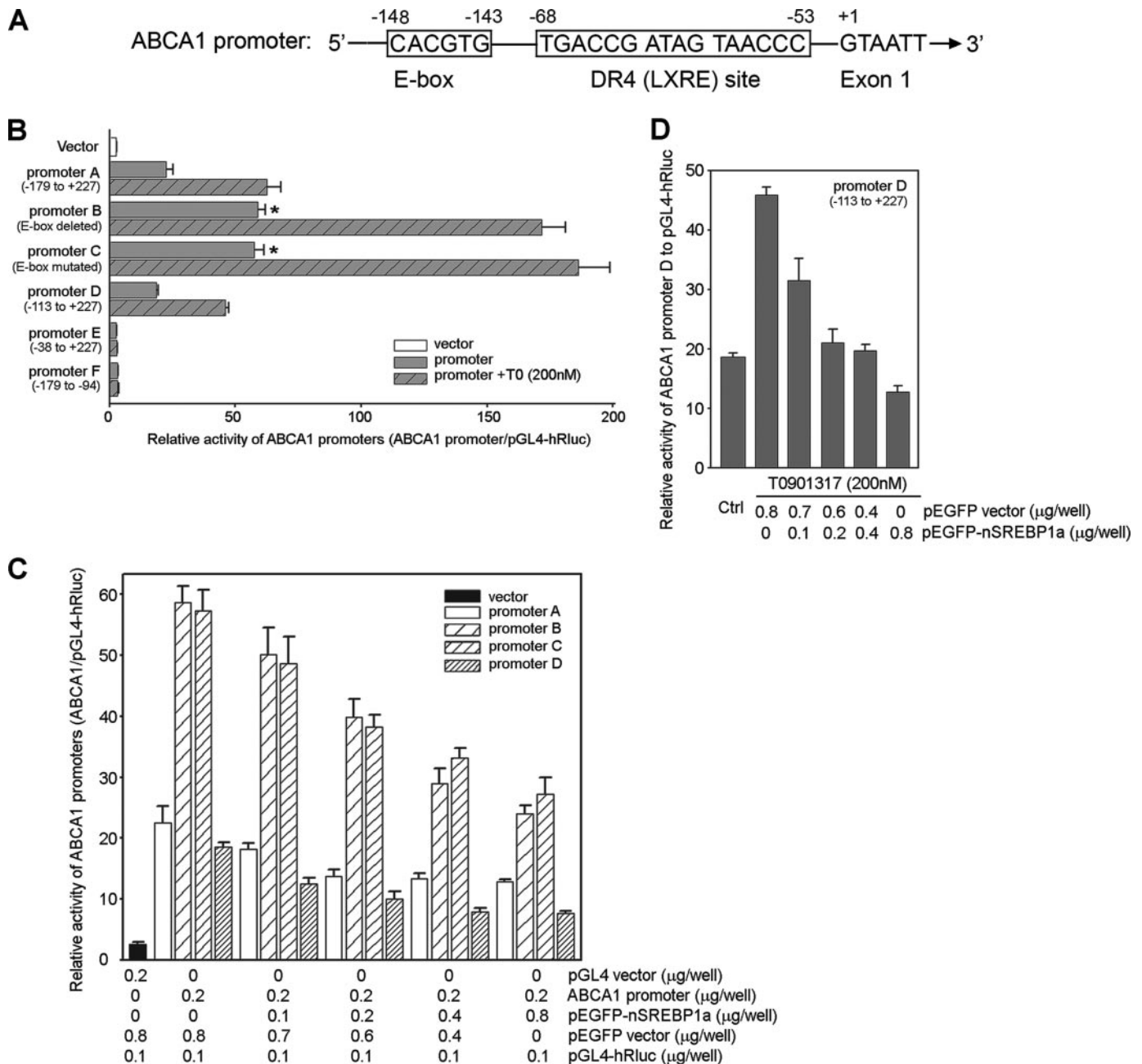


FIGURE 6. Active SREBP1a inhibits ABCA1 promoter activity. A, diagram of mouse proximal ABCA1 promoter including motifs of the E-box and the LXRE or DR4. Exon1 starts with +1. B, determination of activity of various ABCA1 promoters and activation of ABCA1 promoters by LXR ligand (T0901317). 293 cells in 24-well plates were transfected with DNA for ABCA1 promoter A to F plus *Renilla* by using Lipofectamine™ 2000. After 6 h of transfection, cells were treated with 200 nM T0901317 overnight. After washing with PBS, cells were extracted for cellular proteins, and the activity of firefly or *Renilla* luciferase in cellular lysate was determined with the use of the Dual-Luciferase® Reporter Assay System. Relative activity of ABCA1 promoter was normalized to activity of co-transfected pGL4-hRluc. *, significantly different from control of promoter A ($p < 0.05$, $n = 4$). T0901317 induced a significant increase in activity of all promoters except E and F. C, nSREBP1a inhibits ABCA1 promoter activity in a concentration-dependent manner. 293 cells in 24-well plates were co-transfected with DNA for ABCA1 promoter A to D separately and DNA for nSREBP1a at the indicated concentration plus DNA for *Renilla* luciferase. Activity of ABCA1 promoter was determined after 24 h of transfection. Expression of nSREBP1a (in each concentration) resulted in significant reduction in activity of all ABCA1 promoters ($p < 0.05$, $n = 4$). D, nSREBP1a inhibits LXR ligand-induced ABCA1 promoter activity. 293 cells in 24-well plates were co-transfected with DNA for ABCA1 promoter D (excluding the E-box and the sequence surrounding the E-box) at 0.2 μg/well, nSREBP1a at the indicated concentrations, and *Renilla* luciferase. After 6 h of transfection, cells were treated with 200 nM T0901317 overnight followed by determination of ABCA1 promoter D activity. nSREBP1a significantly inhibited T0901317-induced activity of ABCA1 promoter D at all concentrations ($p < 0.05$, $n = 4$). Data from B–D were expressed as the mean \pm S.D.

DISCUSSION

Dysfunction or lack of LDLR expression prevents or reduces hepatic removal of excess cholesterol from the circulation leading to high plasma LDL cholesterol levels. Patients with FH have a high risk of development of early coronary heart disease

(7, 9). Similarly, genetic deletion of the LDLR in mice can result in atherosclerosis (10). Scavenger receptor-mediated binding and internalization of modified (oxidized) LDL is thought to play a major role in the development of atherosclerosis in FH patients and LDLR^{-/-} mice. In this study, we hypothesized that

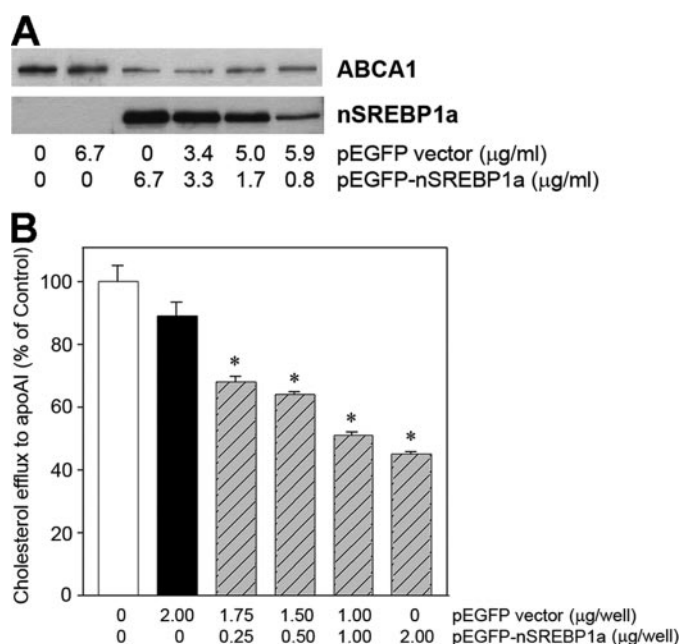


FIGURE 7. nSREBP1a inhibits macrophage ABCA1 expression and cholesterol efflux. A, ~90% confluent RAW264.7 macrophages in 60-mm dishes were transfected with DNA for nSREBP1a at the indicated concentrations. After 24 h of transfection, cells were washed with PBS and used to extract whole cellular proteins. Expression of ABCA1 and nSREBP1a was determined by Western blot. B, prelabeled RAW264.7 macrophages in 12-well plates were transfected with DNA for nSREBP1a or vector as indicated overnight. The effect of nSREBP1a on macrophage free cholesterol efflux to apoA1 (10 μg/ml) was determined. Data were expressed as the mean ± S.D. *, significantly different from control cells or vector DNA alone transfected cells ($p < 0.05$, $n = 4$).

the LDLR may also play a role in regulating cellular cholesterol efflux. In studies described herein we observed that LDLR deletion reduced expression of macrophage ABCA1/ABCG1 and free cholesterol efflux. More importantly, we demonstrated that in wild type macrophages, sterols induced ABCA1 expression, which may reduce lipid accumulation. In contrast, sterols had significant reduced inductive or even inhibitory effects on ABCA1 expression in LDLR^{-/-} macrophages, potentially leading to an imbalance of cholesterol uptake and efflux. Although LDLR deficiency resulted in decreased LDL uptake by macrophages (~80% of wild type cells as determined by fluorescence-activated cell sorter assay), the uptake of cholesterol was unchanged (data not shown). This suggests the reduced induction of ABCA1 expression was not due to the reduced lipid uptake. Furthermore, we discovered that the differential regulation of ABCA1 expression by sterols between wild type and LDLR^{-/-} macrophages was independent of the LXR pathway but dependent on SREBP1 activity.

LXR has a central role in stimulating ABCA1 expression. Oxysterols can function as LXR ligands and induce ABCA1 expression (24, 25). Cholesterol and lipoproteins supply oxysterols to cells that can also activate LXR and ABCA1 expression (45). In contrast, inactivation of LXR by geranylgeranyl pyrophosphate as well as several statins can decrease ABCA1 expression (46, 47). However, our studies suggest that impaired ABCA1 expression in LDLR^{-/-} macrophages is independent of LXR activity since 1) an LXR ligand can affect ABCA1 expression and cholesterol efflux similarly (Fig. 2, A and B), 2) expression of LXRα or LXRβ is similar between the two cell

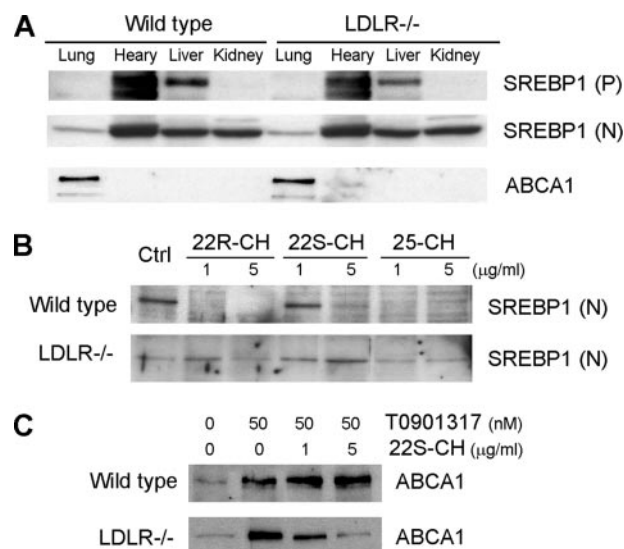


FIGURE 8. Impairment of sterol-induced macrophage ABCA1 expression by LDLR deletion is due to the sterol-activated SREBP1. A, inverse relationship between SREBP1 and ABCA1 expression. Whole cellular proteins or nuclear proteins were extracted from mouse tissues (lung, heart, liver, and kidney). Expression of SREBP1 precursor and nuclear form or ABCA1 protein was determined with nuclear proteins (50 μg) or cellular protein (40 μg) by Western blot. B, oxysterols inhibit SREBP1 in wild type macrophages but have no effect or activate SREBP1 in LDLR^{-/-} macrophages. Wild type and LDLR^{-/-} macrophages in serum-free medium were treated with 22(R)- or 22(S)- or 25-hydroxycholesterol (CH) at 1 or 5 μg/ml overnight. Nuclear proteins were extracted from cells and used to determine the nuclear form of SREBP1 by Western blot. Ctrl, control. C, 22(S)-hydroxycholesterol synergizes with LXR ligand-induced ABCA1 expression in wild type macrophages but blocks the induction in LDLR^{-/-} macrophages. Cells in serum-free medium were co-treated with LXR ligand (T901317, 50 nM) and 22(S)-hydroxycholesterol at the indicated concentrations overnight. Expression of ABCA1 was determined by Western blot.

types (Fig. 2C), and 3) the expression of ABCG1, another target gene of LXR activation, is normally regulated by LXR ligand and lipoproteins (Figs. 2 and 3). In addition, reduction of atherosclerosis in LDLR^{-/-} mice by a synthetic LXR ligand through activation of ABCA1 expression (28) supports our conclusions.

Although LXR functions normally in LDLR^{-/-} macrophages compared with wild type cells, the inductive effects on ABCA1 expression by sterols is significantly reduced in LDLR^{-/-} macrophages, which suggests an inhibitory mechanism may block ABCA1 activation. The role of SREBP2 in regulation of ABCA1 expression is still controversial. In human endothelial cells, Zeng *et al.* (32) reported that SREBP2 negatively influenced ABCA1 expression by binding the E-box in the proximal region of ABCA1 promoter. However, in transfected CHO-7 cells, Wong *et al.* (34) observed that SREBP2 increased ABCA1 expression by a mechanism in which the production of 24(S),25-epoxycholesterol, a potent ligand for LXR, was enhanced, and the E-box did not interfere with the SREBP2-mediated ABCA1 expression. Expression of SREBP1 and SREBP2 is cell type-dependent, and they overlap in functions (35). Expression of SREBP2 is not easily detectable, and expression of SREBP1 is moderate in macrophages, suggesting the potential role of SREBP1 in the regulation of macrophage ABCA1 expression. Indeed, we determined that SREBP1a is a negative regulator of ABCA1 expression in macrophages by several pieces of evidence; 1) inverse patterns of ABCA1 and SREBP1 expression in animal tissues (Fig. 8A), 2) active

SREBP1a inhibits ABCA1 promoter activity, reduces macrophage ABCA1 expression and free cholesterol efflux, and blocks LXR ligand-induced ABCA1 promoter activity (Figs. 6, C and D, and 7), and 3) inhibition of ABCA1 promoter activity by active SREBP1a is dependent on the LXRE, not the E-box (Figs. 6, C and D).

Effects of a sterol on macrophage ABCA1 expression is determined by its effects on activity of LXR and/or SREBP1. In wild type macrophages, sterol may activate LXR as a ligand and simultaneously inhibit SREBP1 by blocking the processing of SREBP1. Either activation of LXR or inhibition of SREBP1 processing can increase ABCA1 expression. 22(R)- and 25-hydroxycholesterol are ligands for LXR (48). In contrast, 22(S)-hydroxycholesterol does not have affinity for LXR (49–51). In addition, effect of 22(S)-hydroxycholesterol on SREBP1 proteolysis is weaker than 22(R)- or 25-hydroxycholesterol (Fig. 8B). Thus, 22(R)- and 25-hydroxycholesterol significantly induces ABCA1 expression, whereas 22(S)-hydroxycholesterol moderately induces ABCA1 expression in wild type macrophages (Fig. 4B). In LDLR^{-/-} cells, the SREBP1 processing in response to sterols is totally different from wild type cells. In fact, 22(R)- or 25-hydroxycholesterol shows a slight inductive effect, whereas 22(S)-hydroxycholesterol demonstrates a moderate inductive effect on SREBP1 proteolysis (Fig. 8B). This activation can inhibit ABCA1 expression. Compared with wild type cells, 22(R)- or 25-hydroxycholesterol display a lesser inductive effect, whereas 22(S)-hydroxycholesterol shows an inhibitory effect on ABCA1 expression in LDLR^{-/-} macrophages (Fig. 4B). The inhibitory pathway on ABCA1 expression by sterols through activation of SREBP1 in LDLR^{-/-} macrophages is further confirmed by the co-treatment of 22(S)-hydroxycholesterol with a synthetic nonsteroid LXR ligand in which 22(S)-hydroxycholesterol synergized with T0901317-induced ABCA1 expression in wild type cells while blocking the induction in LDLR^{-/-} cells (Fig. 8C). Yang *et al.* (52) have proposed that the SREBP processing was not required for sterol-mediated ABCA1 induction since they observed that ABCA1 expression was also induced by sterols through LXR activation in cells lacking the enzymes necessary for SREBP processing. However, results reported herein show that the induction of ABCA1 expression in wild type cells by cholesterol does not occur. Comparable induction of ABCA1 expression by desmosterol or 25-hydroxycholesterol as nonsteroid LXR ligand (T0901317) in wild type cells was also significantly reduced in these mutant cells (52). These results suggest that the inhibition of SREBP activity by sterols in wild type cells can enhance ABCA1 expression mediated by sterol-activated LXR pathway. Similarly in our studies, the lack of LDLR expression results in no SREBP1 processing or activation in response to sterols; thus, the sterol-activated macrophage ABCA1 expression is significantly reduced in LDLR^{-/-} macrophages.

In summary, expression of macrophage ABCA1 is dependent on the activity of LXR and SREBP1. In wild type cells, the inhibition of SREBP1 proteolysis and the activation of LXR by sterols coordinately increase ABCA1 expression and cholesterol efflux. We believe that this may prevent the accumulation of cellular cholesterol. Expression of LDLR is critical to ensure inhibition of SREBP1 proteolysis. Lack of LDLR leads to an

abnormal response of SREBP1 processing to sterols and reduced ABCA1 expression and cholesterol efflux. Therefore, lack of LDLR expression could alter the dynamic balance between cholesterol uptake and efflux, and it may play an unexpected role in the pathogenesis of the atherosclerotic lesions.

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