CHAPTER 2

Hypocholesterolemia, foam cell formation, but no atherosclerosis in mice lacking ABC-transporter A1 and scavenger receptor class B type I

Ying Zhao1, Marieke Pennings1, Carlos L.J. Vrins2, Laura Calpe-Berdiel1, Menno Hoekstra1, J. Kar Kruijt1, Roelof Ottenhoff2, Reeni B. Hildebrand1, Wendy Jessup3, Wilfried Le Goff4, M. John Chapman4, Thierry Huby4, Albert K. Groen5, Theo J.C. Van Berkel1, Miranda Van Eck1

1 Division of Biopharmaceutics, Leiden/Amsterdam Center for Drug Research, Leiden University, Einsteinweg 55, 2333 CC Leiden, The Netherlands
2 Department of Medical Biochemistry, Academic Medical Center, Amsterdam, The Netherlands
3 Centre for Vascular Research, University of New South Wales, Sydney, Australia
4 INSERM U551, Paris, France; Université Pierre et Marie Curie-Paris 06, UMRS 939 Paris, France
5 Department of Pediatrics, University Medical Center Groningen, Groningen, The Netherlands

Abstract

High-density lipoprotein (HDL) mediated reverse cholesterol transport (RCT) is regarded to be crucial for prevention of foam cell formation and atherosclerosis. ABC-transporter A1 (ABCA1) and scavenger receptor BI (SR-BI) are involved in the biogenesis of HDL and the selective delivery of HDL cholesterol to the liver, respectively. In the present study, we phenotypically characterized mice lacking these two proteins essential for HDL metabolism. ABCA1xSR-BI double knockout (dKO) mice showed severe hypocholesterolemia mainly due to HDL loss, despite a 90% reduction of HDL cholesterol uptake by liver. VLDL production was increased in dKO mice. However, non-HDL cholesterol levels were reduced, probably due to enhanced clearance via LRP1. Hepatobiliary cholesterol transport and fecal sterol excretion were not impaired in dKO mice. In contrast, the macrophage RCT in dKO mice was markedly impaired as compared to WT mice, associated with the accumulation of macrophage foam cells in the lung and Peyer’s patches. Strikingly, no atherosclerotic lesion formation was observed in dKO mice. In conclusion, both ABCA1 and SR-BI are essential for maintaining a properly functioning HDL-mediated macrophage RCT, while the potential anti-atherosclerotic functions of ABCA1 and SR-BI are not evident in dKO mice due to the absence of pro-atherogenic lipoproteins.
Introduction

Plasma HDL cholesterol levels are inversely correlated with the risk of atherosclerotic vascular disease [1]. HDL-mediated reverse cholesterol transport (RCT), a process in which excess peripheral cholesterol is removed and delivered to the liver for biliary secretion, has been regarded crucial for prevention of foam cell formation and atherosclerosis [2]. Two important genes recognized in RCT are the ATP-binding cassette transporter A1 (ABCA1) and the scavenger receptor class B type I (SR-BI) [2]. ABCA1 promotes the efflux of cholesterol and phospholipid to lipid-poor apolipoprotein AI (ApoAI), leading to HDL particle maturation, the first step in RCT [2]. In contrast, SR-BI facilitates the bi-directional flux of cholesterol between cells and mature HDL down a concentration gradient. Importantly, SR-BI is essential for the selective uptake of cholesteryl esters (CEs) from HDL by the liver for biliary secretion, the last step in RCT [2].

Recent genome-wide association studies (GWAS) have identified ABCA1 as a major gene influencing HDL levels in humans [3]. Targeted deletion of ABCA1 in the liver [4] and intestine [5] of mice has revealed the crucial roles of hepatic and intestinal ABCA1 for HDL biogenesis. In agreement, induction of hepatic and intestinal ABCA1 expression increases plasma HDL cholesterol (HDL-C) levels [4,6]. In contrast, macrophage ABCA1 minimally contributes to plasma HDL cholesterol [7]. Patients with functional mutations in ABCA1 have Tangier disease (TD), characterized by a marked reduction in plasma HDL-C and increased macrophage foam cell formation in several tissues [8]. Likewise, ABCA1 knockout (KO) mice display the similar pathophysiologic phenotype as TD patients [9]. Cells from patients with TD and ABCA1 KO mice are defective in the efflux of cholesterol and phospholipid to apoAI and partially to HDL [8]. Also, macrophage RCT is impaired in ABCA1 KO mice, mainly due to HDL deficiency [10-11].

In several population studies, a clear association between mutations in the coding and promoter regions of human SR-BI and increased plasma HDL-C has been shown [12,13]. Recent GWAS studies also demonstrated that single nucleotide polymorphism (SNPs) in and near SR-BI are significantly associated with plasma levels of HDL-C in humans [14]. However, only very recently conclusive evidence was provided on the importance of SR-BI for controlling HDL-C levels in humans. Vergeer et al identified a family in which heterozygous carriers of a unique mutation (P297S) in the extracellular domain of SR-BI showed 37% elevated HDL cholesterol levels [15]. Importantly, hepatocytes that expressed the P297S mutant SR-BI displayed a reduced capacity to take up CEs from HDL, thereby leading to the elevation in plasma HDL-C [15]. Also, SR-BI KO mice display increased HDL-C, due to impaired delivery of HDL-CEs to liver [16]. Interestingly, despite the high levels of HDL-C, SR-BI deficient mice have impaired macrophage RCT [17]. Conversely, overexpression of hepatic SR-BI decreases HDL-C and promotes macrophage RCT [17].

Given that ABCA1 and SR-BI are involved in the different steps of RCT, it is conceivable that ABCA1 and SR-BI might act synergistically in the process of RCT. To study the RCT process under conditions in which both of these key mediators are absent, ABCA1/SR-BI double knockout (dKO) mice were generated. In this study, we describe the characterization of ABCA1/SR-BI dKO mice with respect to plasma lipids, HDL metabolism, blood cell counts, bile secretion, macrophage RCT, tissue cholesterol homeostasis, and atherosclerosis susceptibility.
ABCA1xSR-BI double knockout mice

Materials and Methods:

For detailed methodology, please see the data supplement, available online at http://www.sciencedirect.com. Briefly, ABCA1/SR-BI dKO mice were generated by intercrossing double heterozygous offsprings obtained from crossbreeding of ABCA1 KO mice with SR-BI KO mice. Plasma cholesterol levels, the lipoprotein distribution, blood cell counts, and hepatic expression of lipoprotein receptors, including SR-BI, LDL receptor (LDLR), and LDLr related protein 1 (LRP1) were determined. Moreover, VLDL production, serum decay and liver uptake of $[^3]$H-cholesteryl ether-labeled HDL, hepatobiliary and fecal cholesterol secretion, and macrophage RCT were analyzed. In addition, tissue macrophage cholesterol homeostasis and atherosclerosis susceptibility were examined by Oil-red-O staining and immunohistochemical staining against Moma-2.

Results

Hypocholesterolemia and HDL loss in ABCA1/SR-BI dKO mice

To analyze the potential synergistic role of ABCA1 and SR-BI in the RCT process, we generated ABCA1/SR-BI dKO mice. The absence of ABCA1 and SR-BI in the dKO mice was verified at DNA level by performing PCR on genomic DNA (Supplementary Figure 1). From the crosses of ABCA1/SR-BI double heterozygous mice, 7.2%, 6.5%, and 6.2% of the offspring were ABCA1 KO, SR-BI KO, and dKO mice, which are close to the expected Mendelian inheritance rate of 6.25%. Homozygous dKO males are fertile while dKO females, similar to SR-BI KO females [18, 19], are infertile, which could be reversed by administering the cholesterol-lowering drug probucol. However, as described for ABCA1 KO mice [20], also under these conditions a lower frequency of pregnancy and extensive neonatal death of pups born from dKO mothers was observed. No significant differences in body weight between wild-type (WT), ABCA1 KO, SR-BI KO, and dKO mice were observed at the age of 12 weeks (22.7±0.9 g, 20.3±0.3 g, 22.0±0.6 g, 21.3±0.3 g, respectively). Furthermore, dKO mice born from ABCA1 × SR-BI double heterozygous breedings did not appear to have a reduced life expectancy as they can reach ages of >1 year.

Figure 1. Hypocholesterolemia and HDL loss in ABCA1/SR-BI double knockout mice. Total plasma cholesterol levels (A), lipoprotein distribution of total cholesterol (B), HDL cholesterol levels (C), and non-HDL cholesterol levels (D) of WT, ABCA1 KO, SR-BI KO and ABCA1/SR-BI double KO mice (mixed male and female) at the age of 12-16 weeks old on chow. Bar graphs represent the means±SEM (n=8). Two hundred microliters of pooled mouse plasma from the different genotypes were fractioned by FPLC. Statistically significant difference *p<0.05, ***p<0.001 vs WT mice; $$$ p<0.001 vs SR-BI KO mice.
In line with previous studies [8-9,18,19], SR-BI KO mice showed increased plasma free (5.5-fold, p<0.001) and total cholesterol (2.0-fold, p<0.001) levels as compared to WT animals and accumulated abnormally large HDL particles. ABCA1 KO mice on the contrary were severely hypocholesterolemic and had a near complete absence of HDL (Figure 1A-1C and Supplementary Table 4). Mice with a combined deficiency of ABCA1 and SR-BI resembled single ABCA1 KO mice with a dramatic >80% and >99% decrease in the plasma TC and HDL-C levels, respectively (Figure 1A-1C). Of note, ABCA1 deficiency dramatically reduced the plasma free cholesterol levels (22-fold, p<0.001) in the SR-BI KO background (Supplementary Table 4). SR-BI deficiency also led to an increase in plasma levels of phospholipids (1.2-fold, p<0.001) and triglycerides (1.2-fold, p<0.05). In contrast, both ABCA1 KO and dKO mice showed comparably reduced plasma levels of phospholipids (3.0-fold, p<0.001 vs WT) and triglycerides (1.7-fold, p<0.001 vs WT) (Supplementary Table 4).

Figure 2. Reduced serum decay and liver uptake of [3H]CEt-HDL in ABCA1/SR-BI double knockout mice. Serum decay and liver uptake was determined in 12-16 week old female animals on chow diet. (A) The clearance of [3H]CEt-HDL from the circulation in WT (×), ABCA1 KO (Δ), SR-BI KO (∇) and ABCA1/SR-BI double KO (○) mice at 0, 1, 2, 4, and 24 hours after intravenous injection of 200 μg [3H]CEt-HDL. (B) The amount of [3H]CEt-HDL in the circulation and the liver at 4 hours after injection. (C) The amount of [3H]CEt-HDL in the circulation and the liver at 24 hours after injection. Bar graphs represent the means±SEM (n=3/group). Statistically significant difference **p<0.01, ***p<0.001 vs WT mice; ###p<0.001 vs ABCA1 KO mice; $$$ p<0.001 vs SR-BI KO mice.

Serum decay and hepatic uptake of HDL-CE in dKO mice were studied next. Upon injection of [3H]CEt-HDL into WT mice, 60.3±1.3% of the injected label was removed from the blood during the first 4 h (Figure 2A and 2B). Consistent with previous findings [21], serum clearance was severely delayed upon deletion of SR-BI. At 4 h after injection only 35.4±1.9% (p<0.001) was removed from the circulation in SR-BI KO mice. Notably, in ABCA1 KO mice, 87.6±1.4% (p<0.001) of the injected label was removed from the
circulation at 4 h after injection. Combined disruption of ABCA1 and SR-BI led to a significantly delayed serum clearance of [3H]CET-HDL (48.6±1.1% removed at 4h, p<0.001 vs WT mice; p<0.001 vs ABCA1 KO mice). Importantly, these findings in the dKO mice for the first time show that SR-BI deficiency dramatically decreases HDL-CET clearance in the absence of a circulation pool of HDL. At this time point, in WT mice, 26.5±1.5% of the injected dose (ID) was recovered in the liver while ABCA1 KO mice showed a 1.7-fold (46.0±3.1%, p<0.001) increase in uptake by the liver (Figure 2B). In contrast, only 3.5±0.6% (p<0.001 vs WT) and 3.2±0.3% (p<0.001 vs ABCA1 KO) of the ID accumulated in the liver of SR-BI KO and dKO mice, indicating that SR-BI is responsible for the majority (around 80-90%) of the removal of CET from HDL in the circulation by liver. Moreover, the distribution of radioactivity over extrahepatic tissues was also analyzed at 4 hours after injection (Supplementary Table 3). It was found that the majority of the extrahepatic label was present in bone (6-9%), skin (4-9%), and the muscle (1-3%). The total recovery of label was between 79 to 82%. In line with previous study [21], SR-BI deficiency also led to an ~88% (p<0.001 vs WT) reduction of [3H]CET uptake by adrenal. Similarly, the adrenal uptake of [3H]CET in dKO mice was also dramatically decreased 85% (p<0.001) and 97% (p<0.001) as compared to WT and ABCA1 KO mice, respectively (Supplementary Table 3). Interestingly, ABCA1 deficiency led to 6-fold (p<0.001 vs WT) more adrenal uptake of [3H]CET. Also a 3-fold (p<0.01) increase in skin uptake of [3H]CET was observed in ABCA1 KO mice as compared to WT mice. However, the absolute uptake by adrenal and skin in ABCA1 KO mice was only 3.5% and 2.6% of ID. Thus, increased clearance of [3H]CET-HDL from the circulation in ABCA1 KO mice as compared to WT animals was mainly due to enhanced uptake by the liver. After 24 h, above 90% of injected [3H]CET-HDL was removed from the circulation in WT, ABCA1 KO, and dKO mice whereas 30.0±0.8% (p<0.001) of the injected label remained in the circulation of SR-BI KO mice (Figure 2A and 2C). At this time point, 46.0±2.3% and 50.1±3.4% of ID were recovered in the liver of WT and ABCA1 KO mice. In contrast, only 10.2±0.3% (p<0.001 vs WT) and 5.9±0.2% (p<0.001 vs ABCA1 KO) of the ID had accumulated in livers of SR-BI KO and dKO mice, respectively (Figure 2C).

In line with previous findings [22], deletion of SR-BI also resulted in a 1.3-fold (p<0.05) increase in non-HDL-C levels (Figure 1D). Interestingly, non-HDL-C levels were reduced 4.2-fold (p<0.001) and 5.1-fold (p<0.001) in ABCA1 KO and dKO animals, respectively as compared to WT mice (Figure 1D). However, ABCA1 deficiency did lead to a 1.5-fold (P<0.001) and 1.8-fold (P<0.01) higher VLDL production rate against both the WT and SR-BI KO background, respectively (Figure 3A). The observed reduction in non-HDL-C in ABCA1 KO and dKO mice was thus not the result of an impaired hepatic VLDL secretion. Next, we analyzed the hepatic expression of ABCA1, SR-BI, LDLr, and

![Figure 3. Increased VLDL production in ABCA1/SR-BI double knockout mice.](image-url) VLDL production rate was determined in 12-16 week old female animals on chow diet. VLDL production was determined by calculating the accumulation rate (g/h/kg body weight) of triglycerides in the plasma after blocking VLDL clearance by Triton WR-1339. Data are expressed as mean±SEM (n=5/group). Statistically significant difference **p<0.01, ***p<0.001 vs WT mice; $$$p<0.001 vs SR-BI KO mice.
LRP1 at both the mRNA and the protein level. As anticipated, deletion of ABCA1 and/or SR-BI resulted in undetectable mRNA and protein expression of the respective proteins in the liver (Figure 4). ABCA1 deficiency did not affect the expression of hepatic SR-BI. Thus, the observed increased accumulation of $[^3H]$CEt-HDL in the liver of ABCA1 KO mice might be due to the lack of an endogenous HDL pool, which could compete with $[^3H]$CEt-HDL in binding with hepatic SR-BI. The mRNA levels of LDLr and LRP1 were increased 2.5-fold ($p<0.01$) and 2.0-fold ($p<0.01$), respectively in the liver of dKO mice as compared to WT and single KOs (Figure 4A). However, only the protein levels of hepatic LRP1 were significantly upregulated 1.7-fold ($p<0.05$) in the dKOs (Figure 4B). Strikingly, the protein levels of the LDLr in livers of the dKO mice were dramatically reduced (4-fold, $p<0.05$) (Figure 4B). Since dKO mice showed 3-fold ($p<0.01$) higher hepatic expression of PCSK9 protein as compared to WT mice and single KOs (Supplementary Figure 2A), the reduced LDLr protein levels in the liver might be due to enhanced degradation of the LDLr via PCSK9 [23] (Supplementary Figure 2A). In addition, the expression of P2Y13, an important player in HDL metabolism [24], was also analyzed by Western blot. As shown in Supplementary Figure 2B, single ABCA1 and SR-BI deficiency did not affect the expression of P2Y13 in the liver. In contrast, dKO mice showed slightly increased hepatic levels of P2Y13 (1.5-fold, $p<0.05$ vs WT mice and single KOs).

![Figure 4](image-url)

**Figure 4. Expression levels of ABCA1, SR-BI, LDLr, and LRP1 in livers of ABCA1/SR-BI dKO mice.** Livers were harvested from 16-week old chow-fed WT, ABCA1 KO, SR-BI KO, and dKO mice and total RNA and protein were isolated for real-time PCR analysis (A, n=6) and western blotting analysis (B, n=3), respectively as described in Materials and Methods. The expression levels in livers of WT animals were normalized to 1. Data are expressed as mean±SEM. Statistically significant difference *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs WT mice; *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs ABCA1 KO mice; *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs SR-BI KO mice.
Normalized red blood cell and reticulocyte counts in ABCA1/SR-BI dKO mice

Circulating white and red blood cells were next analyzed. No significant differences in white blood cells, including lymphocytes, monocytes, and granulocytes were observed among the different strains of animals (Supplementary Table 5). In line with previous study [25, 26], SR-BI KO mice showed a slightly reduced red blood cell (RBC) count (10.0±0.1 x10^9/mL vs 11.1±0.2 x10^9/mL in WT mice, p<0.01) with an increased reticulocyte count (215±15‰ vs 34±1‰ in WT mice, p<0.0001) (Supplementary Table 5). No significant differences in the RBC (11.5±0.2 x10^9/mL) and reticulocyte (36±2‰) count were found in ABCA1 KO mice as compared to WT mice. Notably, ABCA1 deficiency normalized the RBC and reticulocyte counts in SR-BI KO mice (dKO mice: RBC, 10.8±0.3 x10^9/mL, p<0.05; reticulocyte, 38±5‰, p<0.001 vs SR-BI KO). This is most likely the consequence of the large reduction in FC levels in the dKO mice [25,26].

Unaltered hepatic lipid content and biliary and fecal sterol secretion in ABCA1/SR-BI dKO mice

Hepatic total cholesterol, phospholipids, and triglycerides levels were not changed in dKO mice as compared to WT and single KOs (Supplementary Table 6). In line with one previous study [27], ABCA1 deficiency did not influence biliary cholesterol secretion. Also, the biliary secretion rate of cholesterol was not impaired in dKO mice, although a slight but not significant decrease in biliary cholesterol secretion was observed in SR-BI KO mice. The expression of ABCG5 and ABCG8, important cholesterol transporters on hepatic canalicular membrane [28], was unaffected in SR-BI KO and dKO mice (Supplementary Figure 2C and 2D). Moreover, biliary secretion of phospholipid and bile salts were lower in dKO mice, but the differences were not statistically significant, which might be due to the high variation among the animals (Supplementary Table 6). Furthermore, dKO mice produced similar amount of feces (1.26±0.35 g/day) as compared to WT mice (1.13±0.08 g/day). Lipid analysis of fecal samples showed that combined deficiency of ABCA1 and SR-BI did not affect fecal cholesterol (2.34±0.04 vs 2.43±0.09 mmol/kg) and bile salt content (12.75±2.28 vs 10.30±0.65 mmol/kg) as compared to WT mice. Thus, despite the absence of HDL, ABCA1 deficiency did not alter the hepatic lipid content and biliary and fecal sterol secretion in either the WT or the SR-BI KO background.

Impaired macrophage RCT in ABCA1/SR-BI dKO mice

Next, we investigated the effect of combined deficiency of ABCA1 and SR-BI on macrophage-specific RCT. First, bone marrow-derived macrophages from WT, ABCA1 KO, SR-BI KO, and dKO mice were loaded with [3H]-cholesterol and acetylated LDL (acLDL). The lipid-laden macrophages were subsequently injected intraperitoneally into WT mice, and the transport of labeled cholesterol to blood, liver, and feces was measured at 24 h after the injection. Consistent with previous findings [11], ABCA1 deficiency on macrophages did result in a ~20% reduction (p<0.05) in the amount of tracer excreted into feces. However, given that the expression of macrophage SR-BI was undetectable after cholesterol loading, we could not pick up the effect of macrophage SR-BI deficiency on RCT in this experimental setting (Supplementary Figure 3). Thus, we next quantified the release of [3H]-cholesterol from acLDL-loaded WT macrophages to the plasma and their transport to the liver and ultimately the feces in WT, ABCA1 KO, SR-BI KO and dKO mice. In line with previous findings [10,11,17], at 24 hours after injection, the absence of HDL due to ABCA1 deficiency resulted in a significant reduction of 3H-cholesterol in
plasma (13.5-fold, p<0.001), liver (1.5-fold, p<0.05), and feces (1.3-fold, p<0.05) (Figure 5). SR-BI KO mice with impaired hepatic uptake of HDL-CE had significantly increased \(^3\)H-cholesterol (1.7-fold, p<0.001) in the plasma but reduced \(^3\)H-cholesterol in the liver (1.4-fold, p<0.05) and feces (1.3-fold, p<0.05) (Figure 5). Combined deficiency of ABCA1 and SR-BI also led to a significant reduction of \(^3\)H-cholesterol in the plasma (13.3-fold, p<0.001), liver (1.7-fold, p<0.05), and feces (1.4-fold, p<0.05). However, no added effect of ABCA1 and SR-BI on the reverse transport of cholesterol from WT macrophages was observed.

**Figure 5.** Impaired macrophage-specific reverse cholesterol transport in ABCA1/SR-BI double knockout mice. Peritoneal macrophages from WT mice were prepared and labeled with \[^{3}H\]-cholesterol and acetylated LDL (5 \(\mu\)Ci/mL, 100 \(\mu\)g/mL) for 48 hours. After equilibration, \[^{3}H\]-cholesterol labeled macrophage foam cells were harvested and injected into male WT, ABCA1 KO, SR-BI KO and ABCA1/SR-BI double knockout mice at the age of 12-16 weeks old on chow. Blood (A), liver (B), and feces (C) were collected at 24 hours after injection and the tracer levels in various samples were measured. Data are expressed as percentage of the \[^{3}H\]-cholesterol tracer relative to total dpm tracer injected ±SEM (n=6). *p<0.05, **p<0.01, ***p<0.001 vs WT mice; ###p<0.001 vs SR-BI KO mice.

**Tissue cholesterol homeostasis in ABCA1/SR-BI dKO mice**

To assess the morphological changes associated with combined ABCA1 and SR-BI deficiency, a necropsy of the mice at the age of 16 weeks old on chow diet was performed. No significant differences were observed in liver weight between WT, ABCA1 KO, SR-BI KO, and dKO mice (50.0±1.8 mg/g, 55.3±2.1 mg/g, 56.0±3.0 mg/g, 52.5±3.3 mg/g body weight, respectively). In line with previous findings [8,25,26], ABCA1 KO mice and SR-BI KO mice showed a 1.3-fold (4.4±0.2 mg/g, p<0.05) and a 3.3-fold (11.0±0.5 mg/g, p<0.001) increase in spleen weight, respectively as compared to WT mice (3.3±0.2 mg/g body weight). Interestingly, the spleen weight of dKO mice was increased 1.9-fold to 6.3±0.7 mg/g body weight (p<0.001 vs WT), which was significantly lower than the spleen weight of SR-BI KO mice (1.7-fold, p<0.001) and higher than the spleen weight of ABCA1 KO mice (1.4-fold, p<0.01). The splenomegaly observed in SR-BI KO mice was associated with enhanced erythropoiesis in the spleen and the accumulation of reticulocytes in the blood, due to the abnormally high plasma FC levels [25,26]. ABCA1 deficiency did lower plasma FC levels and normalized the counts of reticulocytes in the circulation of SR-BI KO mice (Supplementary Table 4 and 5). The splenomegaly observed in the dKO mice thus can not be the result of enhanced erythropoiesis.

To further determine the effect of combined deficiency of ABCA1 and SR-BI on tissue cholesterol homeostasis, cryostat sections of liver, spleen, lung, thymus, and Peyer’s patches in mice at the age of 16 weeks old on chow were stained for neutral lipids with
Oil-red-O. Interestingly, no visible lipid accumulation was found in liver, spleen, and thymus in WT, single ABCA1 KO, single SR-BI KO, and dKO mice at the age of 16 weeks (data not shown). Thus, the splenomegaly observed in single and double KOs was also not due to lipid accumulation. Consistent with previous findings [9], neutral lipid accumulation was observed in the lung of the single ABCA1 KOs. Also, Peyer’s patches of ABCA1 KOs showed red staining with Oil-red-O, consistent with lipid accumulation. Strikingly, dKO mice displayed more extreme macrophage foam cell formation in the lung and Peyer’s patches as evidenced by colocalization of Oil-red-O with Moma-2 macrophage staining (Figure 6A). Lipid quantification indicated that accumulated neutral lipid in the lung is cholesterol ester (dKO: 204.2±31.04 μg/mg protein vs WT: 1.8±1.2 μg/mg protein, p<0.001). Furthermore, atherosclerosis was analyzed at the aortic root of separate mice at the age of 1 year old on chow diet. As shown in Figure 6B, no atherosclerotic lesions were evident in the WT, ABCA1 KO, and SR-BI KO mice. Despite enhanced macrophage foam cell formation in the lung and Peyer’s patches, also dKO mice did not develop any atherosclerotic lesions, even at the age of 1 year. This is most likely due to the fact that the dKO mice lack the high plasma levels of atherogenic lipoproteins needed to trigger arterial wall accumulation of macrophages.

**Discussion**

In the current study, we for the first time phenotypically characterized ABCA1/SR-BI dKO mice, and studied the interrelationship between ABCA1 and SR-BI in macrophage RCT
using these unique dKO mice lacking both transporters essential for HDL metabolism. ABCA1/SR-BI dKO mice resembled single ABCA1 KO mice in HDL loss and hypocholesterolemia. Although the transport of cholesterol from WT macrophages to feces was impaired in dKO mice, combined deficiency of ABCA1 and SR-BI did not result in an additive effect as compared to the rate of cholesterol transport to feces in single ABCA1 KO and SR-BI KO mice. Interestingly, enhanced macrophage foam cell formation was evident in the lung and Peyer’s patches of dKO mice, clearly illustrating the importance of both ABCA1 and SR-BI for macrophage cholesterol homeostasis in these organs. However, no atherosclerotic lesion development was observed in these dKOs, even at the age of 1 year, probably due to the low levels of non-HDL-C.

SR-BI mediated hepatic uptake of HDL cholesterol is nearly completely blocked in SR-BI KO and dKO mice, similar as in SR-BI KO mice. However, with respect to the lipid levels in the plasma and the lipoprotein distribution of cholesterol, our dKO mice resembled single ABCA1 KO mice. The HDL loss in the dKO mice is thus due to impaired HDL production, similarly as previously described for single ABCA1 KO mice [29]. Although ABCA1 deficiency did not affect the hepatic expression of SR-BI, ABCA1 KO mice did take up 2 times more [3H]-CET from HDL by liver as compared to WT mice. This clearly indicates that endogenous HDL could compete with [3H]-CET-HDL for uptake by the liver, thereby inhibiting the clearance of [3H]-CET-HDL in wild-type mice. Importantly, the absence of the endogenous HDL pool in ABCA1 KO and dKO mice allowed us to study the role of SR-BI in HDL catabolism in absence of competition with endogenous HDL in the circulation. We hereby thus for the first time provide direct compelling in vivo evidence that SR-BI is the determining factor for the selective uptake CE from HDL by liver in the absence of the endogenous HDL pool.

Interestingly, dKO mice also resembled the single ABCA1 KOs in non-HDL-C levels, which were significantly lower than WT and SR-BI KO mice. VLDL production, however, was increased in dKO and single ABCA1 KO mice. Chung et al previously demonstrated that pre-β migrating nascent HDL generated by ABCA1 inhibits VLDL production through activation of phosphoinositide 3 (PI3)-kinase [30]. As a result, ABCA1 deficiency leads to increased VLDL production in vivo secondary to reduced PI3-kinase signaling [31]. Moreover, SR-BI has been shown to promote VLDL production through induction of microsomal triglyceride transfer protein (MTP) activity [32], which might also be regulated via PI3-kinase signaling [33]. In the present study and previous studies [22], we could not show the effect of SR-BI deficiency on VLDL production. SR-BI KO mice, however, do display increased non-HDL cholesterol levels which can be attributed to a direct role of SR-BI in the clearance of VLDL [22]. In the absence of competition with endogenous HDL, VLDL clearance via SR-BI thus might also contribute to the lower levels of non-HDL-C in ABCA1 KO mice. In contrast, the decreased non-HDL-C levels in the circulation of dKO mice are probably the consequence of compensatory upregulation of LRP1-mediated clearance pathway in the liver.

In agreement with a previous study showing that biliary cholesterol secretion is not affected by the absence of HDL [27], our single ABCA1 KO and dKO mice did not display impaired biliary cholesterol secretion. The reduced biliary cholesterol secretion in SR-BI KO mice was observed in previous studies [34,35,36], which could not be confirmed in the current study. Differences in the background of mice [34,35] and treatment of mice, e.g. dietary challenge[35] and viral exposure [36], between the present study and previous studies might account for this different observation. The low levels of basal biliary cholesterol secretion in our single SR-BI KO and dKO mice indicate that SR-BI might not be essential for the basal cholesterol secretion or that the other cholesterol secretion pathways such as ABCG5 and ABCG8 are redundant under this condition.
Although overexpression of SR-BI could increase biliary secretion in the absence of ABCG5 and ABCG8 [37], our results indicate that the contribution of SR-BI at the physiological level to the biliary cholesterol secretion still needs further investigation.

Despite being a tiny pool for RCT, macrophage RCT is crucial for the prevention of macrophage foam cell formation and atherosclerosis [38]. Inactivation of ABCA1 results in HDL deficiency, thereby impairing macrophage RCT [10-11]. Blocking the hepatic CE uptake from HDL by the deletion of SR-BI also impairs macrophage RCT [17]. In line, HDL deficiency in ABCA1/SR-BI dKO mice did lead to the reduced transport of the [3H]-cholesterol tracer from WT macrophages to feces. SR-BI deficiency, however, did not further impair macrophage RCT in the dKO mice as compared to single ABCA1 KO mice. Thus, in dKO mice, the effect of ABCA1 on generation of HDL rather than the delivery of HDL-C to the liver via SR-BI is the rate limiting factor for macrophage RCT. Notably, in ABCA1/SR-BI dKO mice, a substantial amount of the [3H]-cholesterol tracer could still be transferred from WT macrophages to the feces. [3H]-Cholesterol from the macrophages is detected in plasma not only in the HDL pool but also in the non-HDL pool [10-11]. Therefore, non-HDL lipoproteins might also participate in macrophage RCT. Augmentation of the clearance of non-HDL lipoproteins by liver might be thus important for promoting macrophage RCT, thereby protecting against foam cell formation and atherosclerosis. In line, macrophage-derived apoE promotes both the clearance of non-HDL lipoproteins [39] and macrophage-specific reverse cholesterol transport in apoE KO mice [40]. Moreover, erythrocytes and albumin might also aid the transport of cholesterol through the circulation [41,42]. In addition, Van der Velde et al reported a novel reverse cholesterol transport pathway, namely transintestinal cholesterol transport (TICE) that directly transfers cholesterol from blood to the intestinal lumen via enterocytes. TICE can contribute for up to 70% of the daily total body neutral sterol secretion in mice [43]. Interestingly, TICE is not mediated through HDL particles because ABCA1 KO and our dKO mice show unaltered TICE (Vrins CLJ, unpublished data). How important TICE is for macrophage RCT, however, remains to be determined.

ABCA1/SR-BI dKO mice display enhanced macrophage foam cell formation in the lung and Peyer’s patches. Macrophages cannot limit the uptake of cholesterol and thus depend on cholesterol efflux mechanisms to prevent foam cell formation. ABCA1, ABCG1, and SR-BI are important players in cholesterol efflux from macrophages [44]. ABCA1 and ABCG1 actively transfer cholesterol to lipid-free/poor apoAI and mature HDL, respectively while SR-BI induces cholesterol efflux to a phospholipid-containing acceptor like mature HDL down a concentration gradient. HDL deficiency and reduced macrophage RCT in single ABCA1 KO and dKO mice thus contribute to the macrophage foam cell formation observed in the lung and the Peyer’s patches. Interestingly, however, since more extreme foam cell formation was observed in dKO mice as compared to ABCA1 KO mice, probably also absence of ABCA1 and SR-BI in the macrophages will have contribution to the observed foam cell formation. Wang et al recently demonstrated that macrophage SR-BI does not promote macrophage RCT [45]. In the present study, we also failed to pick up the effect of macrophage SR-BI on macrophage RCT. However, cholesterol loading in vitro does downregulate the expression of SR-BI on WT macrophages [46]. Importantly, in previous bone marrow transplantation studies, we have shown that combined deficiency of macrophage ABCA1 and SR-BI did lead to massive foam cell formation in the peritoneal cavity and spleen of LDLr KO mice transplanted with dKO bone marrow and challenged with a Western-type diet [47]. Thus, the combined role of ABCA1 and SR-BI in cholesterol efflux could account for the enhanced formation of macrophage foam cells in the lung and Peyer’s patches of the dKO mice in vivo. Interestingly, ABCA1/ABCG1 dKO mice showed even more extreme foam cell formation.
in the peritoneal cavity as well as other macrophage-rich tissues, including liver, spleen, lung, Peyer’s patches and thymus [48]. Thus, despite the similarity in acceptor specificity of SR-BI and ABCG1, their relative \textit{in vivo} importance could be affected by the biological environment of the macrophage and possibly the availability of substrates [47]. Interestingly, both ABCA1/ABCG1 dKO mice and ABCA1/SR-BI dKO mice show enhanced foam cell formation under hypocholesterolemia. The source of lipids for the observed foam cell formation is still elusive. Recent findings indicate that Toll-like receptor activation induces macrophage foam cell formation by promoting the lipid droplet formation in the absence of extracellular lipids [49]. Whether this mechanism contributes to the foam cell formation in our dKO mice warrants further investigation in future. Of note, like ABCA1/ABCG1 dKO mice [48], ABCA1/SR-BI dKO mice did not develop atherosclerotic lesions, further indicating the importance of high levels of atherogenic lipoproteins for the development of atherosclerosis.

In summary, by characterizing our unique ABCA1/SR-BI dKO mice, we have provided more insight into the important roles of ABCA1 and SR-BI in lipid metabolism and macrophage foam cell formation \textit{in vivo}. Our data indicate that both ABCA1-mediated HDL generation and HDL-CE delivery to the liver via SR-BI are essential for macrophage RCT. Macrophage foam cells can be formed under hypocholesterolemia, while the presence of high levels of atherogenic lipoproteins is essential for the induction of atherosclerotic lesion formation.

\textbf{Acknowledgements}

This work was supported by grants from the Netherlands Heart Foundation (2001T4101 to M.V.E. and Y.Z., 2008T070 to M.H., and the Established Investigator grant 2007T056 to M.V.E.), Dutch Digestive Foundation (MLDS grant 04-55; CV), the Catalan University and Research Grants Management Agency (Beatriu de Pinós Postdoctoral Grant to L.C-B), and the Netherlands Organization for Scientific Research (VIDI grant 917.66.301 to M.V.E.).

\textbf{References}


37. Zhao Y, Marcel YL. Serum albumin is a significant intermediate in cholesterol transfer between cells and lipoproteins. *Biochemistry.* 1996; 35:7174-7180


Supplementary methods and data

Materials and Methods

Animals

Both ABCA1 knockout (ABCA1 KO) and SR-BI knockout (SR-BI KO) mice, kindly provided by Dr. G. Chimini [1] and Dr. M. Krieger [2], respectively, were back-crossed onto a C57Bl/6 background for at least 8 generations. Double heterozygous offsprings generated by cross-breeding were subsequently intercrossed to obtain the ABCA1/SR-BI double knockout (dKO) mice. The genotypes were analyzed using the primers shown in supplementary table 1. Mice were maintained on sterilized regular chow, containing 4.3% fat and no added cholesterol (RM3; Special Diet Services, Witham, UK). Animal experiments were performed at the Gorlaeus Laboratories of the Leiden/Amsterdam Center for Drug Research in accordance with the National Laws. All experimental protocols were approved by Ethics Committee for Animal Experiments of Leiden University.

Plasma and hepatic lipid analyses

After an overnight fasting-period, 200 μL of blood was drawn from the mice by tail bleeding. Hepatic lipids were extracted according to the method of Bligh & Dyer [3] and dissolved in 2% Triton X-100. Triglycerides (TG) and Phospholipids (PL) in serum and liver were determined using a standard enzymatic colorimetric assay (TG: Roche Diagnostics, Mannheim, Germany; PL: Spinreact, Girona, Spain). The concentrations of cholesterol in serum and liver were determined by incubation with 0.025 U/mL cholesterol oxidase (Sigma), 0.065 U/mL peroxidise, and 15 μg/mL cholesteryl esterase (Roche Diagnostics, Mannheim, Germany) in reaction buffer (1.0 KPi buffer, pH=7.7 containing 0.01 M phenol, 1 mM 4-amino-antipyrine, 1% polyoxyethylene-9-laurylether, and 7.5% methanol). Absorbance was read at 490 nm. The hepatic lipids levels were normalized to their protein concentrations determined using the BCA™ protein assay (Pierce Biotechnology, Rockford, USA). HDL cholesterol was measured after precipitation with phosphotungstic acid and magnesium ions (Boehringer GmbH). The distribution of cholesterol over the different lipoproteins in serum was determined by fractionation of 30 μL of serum of the individual mice using a Superpose 6 column (3.2 x 300 mm, Smart system; Pharmacia, Uppsala, Sweden).

Blood cell count

Blood was drawn from the tail vein of mice on chow diet at the age of 12-16 weeks old. White and Red blood cells were quantified using an automated Sysmex XT-2000iV Veterinary Hematology analyzer (Sysmex Corporation, Kobe, Japan).
Chapter 2

Analysis of gene expression by real-time quantitative PCR

Total RNA was extracted from liver of male animals at the age of 16 weeks old on chow by the acid guanidium thiocyanate-phenol chloroform extraction method according to Chomczynski et al [4]. cDNA was synthesized from 1 μg of total RNA using RevertAid™ M-MuLV Reverse Transcriptase according to manufacturer’s instructions. mRNA levels were quantitatively determined on an ABI Prism® 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) using SYBR-green technology. PCR primers (Supplementary Table 2) were designed using Primer Express 1.5 Software with the manufacturer’s default settings (Applied Biosystems). mRNA expression levels are indicated relative to the average of the housekeeping genes hypoxanthine phosphoribosyltransferase (HPRT), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ribosomal protein 36B4, and β-actin.

Western blot Analysis

Livers from 16-week old chow-fed male animals were homogenized with T-PER tissue protein extraction reagent with added protease inhibitors (Pierce Biotechnology, Rockford, USA). Cell debris was removed by centrifugation at 12,000 rpm for 10 min, and the protein content was determined by the Bio-Rad protein assay (Pierce Biotechnology). Aliquots of 50 μg of protein were separated by 7.5% SDS-PAGE electrophoresis and transferred onto nitrocellulose membranes (Schleicher and Schnell, Dassel, Germany). Immunolabelling was performed using either the rabbit polyclonal antibody α-SR-BI (ab3; Abcam, Cambridge, UK), rabbit polyclonal α-LDL receptor-related protein type 1 (α-LRP-1) (H-80; Santa Cruz Biotechnology, CA), rabbit polyclonal α-LDL receptor (EP1553Y) and α-P2Y13 (Novus Biologicals, Cambridge, UK), rabbit polyclonal α-PCSK9 (ab31762, Abcam, Cambridge, UK), rabbit polyclonal α-ABCG5 and α-ABCG8 (H-300, Santa Cruz Biotechnology), mouse monoclonal antibody α-β-actin (C4; Santa Cruz Biotechnology), or mouse monoclonal α-ABCA1 (AC-10, kindly provided by Dr M. R. Hayden (Vancouver, Canada)) as primary antibodies and horseradish peroxidase-conjugated goat-anti-rabbit and goat-anti-mouse IgG (Jackson ImmunoResearch), respectively, as secondary antibodies. Finally, immunolabeling was detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology).

Hepatic HDL cholesterol uptake assay

Human HDL was isolated from blood of healthy subjects by differential ultracentrifugation as described by Redgrave (1975). HDL (1.063 g/mL<d<1.21 g/mL) was labeled with [3H]-Cholesteryl oleoyl ether (CEt) via exchange from donor particles as reported previously [5]. Unilamellar liposome donor particles were formed by sonication of egg yolk phosphatidylcholine supplemented with 50 μCi of [3H]-CEt. A dose of 200 μg apolipoprotein (± 1.2 × 10^6 dpm) of [3H]CEt-HDL (total volume of 100 μL) was injected into the tail vein of the indicated types of female mice at the age of 12-16 weeks old on chow. At 3 min after injection, a blood sample was drawn to verify the injected dose. At 1h, 2h, 4h, and 24h after injection, blood samples were drawn to measure serum decay. For analysis of liver association, the liver was excised at 24 h after tracer injection, weighed, solubilized, and counted for [3H] radioactivity in a Packard liquid scintillation unit. A correction was made for the radioactivity in the blood present in the liver at the time of sampling as determined by injection of screened [125I]BSA (84.7 μL/g wet weight). Recovery of radioactivity in tissues is around 80% in all different strains of mice (Supplementary Table 3).

In-vivo VLDL production

Female animals at the age of 12-16 weeks old on chow were injected intravenously with 500 mg of Triton WR-1339 (Sigma) per kg body weight as a 15 g/dL solution in 0.9% NaCl after an overnight fast. Previous studies have shown that plasma VLDL clearance is virtually completely inhibited under these conditions [6]. Blood samples (50 μL) were taken at 0, 1, 2, and 3h after Triton WR-1339 injection. Plasma triglycerides were analyzed enzymatically as described above and were
related to the body mass of the animals. The hepatic VLDL production was calculated from the slope of the curve and expressed as g/h/kg body weight.

**Bile sampling and biliary Lipid Content determination**

Bile was collected from 12-16 weeks old chow-fed male mice by cannulation of the gallbladder under hypnorm (fentanyl/fluanisone; 1 mg/kg) and diazepam (10 mg/kg) anaesthesia, using a humidified incubator to maintain body temperature. Bile was collected at 15 min intervals, and production was determined gravimetrically. All collected bile samples were examined for bile salt and lipid content as described previously [7-8].

**Fecal sterol secretion**

The male animals at 12-16 weeks old on chow were placed individually in a metabolic cage with food and water available ad libitum. Feces were collected for 24 hours. Fecal samples were lyophilized, weighed and homogenized. Neutral sterols and bile salts were analyzed by gas liquid chromatographic procedures [8].

**In-vivo macrophage-specific reverse cholesterol transport assay**

Thioglycollate-elicited peritoneal macrophages (PMs) from wildtype mice were loaded with 5 μCi/mL [1α,2α(α-3H]-cholesterol (Amersham Biosciences Europe GmbH, Germany) and 100 μg/mL of acetylated LDL (acLDL) in DMEM/0.2% BSA for 48 hours, while bone marrow-derived macrophages (BMMs) from different genotypic mice were loaded with 50 μg/mL [3H]-cholesterol-labeled acLDL (5 μCi/mL) in fresh differentiation media containing 4 μg/mL 22-hydroxycholesterol and 1 μmol/L 9-cis retinoic acid for 48 hours. These cells were washed, equilibrated and harvested by treatment with accutase (PAA) for 15 min at 37°C. Radioactivity incorporated in the macrophages was determined by lipid extraction and liquid scintillation counting (Beckman). To determine the effect of combined deletion of macrophage ABCA1 and SR-BI on RCT, 5x10⁶ 3H-cholesterol labeled-BMMs in 0.5 mL PBS were injected intraperitoneally into wildtype male mice on chow at the age of 12-16 weeks old. Next, the different genotypic male mice on chow at the age of 12-16 weeks old were injected intraperitoneally with 2×10⁶ 3H-cholesterol labeled-PMs in 0.5 mL PBS to determine the effect of combined hepatic ABCA1 and SR-BI deficiency on RCT. Blood was collected at 24 hours after injection via the vena cava and plasma was used for liquid scintillation counting (Beckman). Livers were removed, weighed, solubilized, and counted in a scintillation vial. Feces were collected, dried at 50°C, weighed and rehydrated in 10 mL miliQ water overnight. Fecal samples were then homogenized and radioactivity was determined by liquid scintillation counting (Beckman). The amount of ³H-tracer in plasma, liver, and feces was expressed as a percent of the injected dose.

**Histological and tissue lipid analysis**

Histological analyses were performed on 10-μm cryosections of lung, liver, spleen, thymus, and Peyer’s patches from 16-week old chow-fed animals. Lipid accumulation was determined by oil red O staining, combined with hematoxylin to visualize nuclei. Macrophages were visualized by immunolabeling against Moma-2 (Research diagnostics). Novared was used as the substrate. The red color is the positive staining, visible with regular light microscopy. Atherosclerotic lesion development was quantified in the aortic root of dKO mice of 1 year old animals on chow as described before [9].

**Statistical Analysis**

Values are expressed as mean±SEM. A one way ANOVA and the Student Newman Keuls posttest were used to compare means after confirming normal distribution by the method Kolmogorov and Smirnov using Graphpad Instat Software (San Diego, USA). A p value of <0.05 was considered significant.
Supplementary data

**Supplementary Figure 1.** Verification of the ABCA1/SR-BI dKO mice. PCR analysis of genomic DNA to verify the genotype of wildtype (WT), ABCA1 knockout (KO), SR-BI KO, and ABCA1/SR-BI double KO (dKO) mice.

**Supplementary Figure 2** Hepatic protein levels of PCSK9 (A), P2Y13 (B), ABCG5 (C), and ABCG8 (D) in ABCA1/SR-BI dKO mice. Livers were harvested from 16-week old chow-fed WT, ABCA1 KO, SR-BI KO, and dKO male mice and total protein was isolated for Western blot analysis as described in Materials and Methods. The expression levels in livers of WT animals were normalized to 1. Data are expressed as mean±SEM (n=3). Statistically significant difference *p<0.05 and **p<0.01 vs WT mice; *p<0.05 and **p<0.01 vs ABCA1 KO mice; *p<0.05 and **p<0.01 vs SR-BI KO mice.
Supplementary Figure 3 Effect of combined deletion of macrophage ABCA1 and SR-BI on reverse cholesterol transport. (A) Macrophages from WT and SR-BI KO mice were labeled with 50 μg/mL [3H]-cholesterol-labeled acLDL before intraperitoneal injection into male WT mice on chow at the age of 12-16 weeks old. (B) Macrophages from WT, ABCA1 KO, and ABCA1/SR-BI dKO mice were labeled with 50 μg/mL [3H]-cholesterol-labeled acLDL in the presence of 4 μg/mL 22-hydroxy-cholesterol and 1 μmol/L 9-cis retinoic acid before intraperitoneal injection into male wildtype mice on chow at the age of 12-16 weeks old. Quantification of macrophage-specific RCT was determined at 24 hours after injection. Data are expressed as mean±SEM (n=4-6/group). Statistically significant difference *p<0.05. Macrophage SR-BI deficiency did not affect macrophage-specific RCT, while macrophage ABCA1 deletion led to reduced macrophage-specific RCT. Combined deletion of ABCA1 and SR-BI led to a similar reduction of macrophage-specific RCT as single ABCA1 deficiency.

Supplementary Table 1: Primers for detection of both wild type and the disrupted ABCA1 and SR-BI gene

<table>
<thead>
<tr>
<th></th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>PCR products (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCA1 WT</td>
<td>5'-TGGGAACCTCTGCTAA AAT-3'</td>
<td>5'-CCATGTGGGTGTGTA CA-3'</td>
<td>600</td>
</tr>
<tr>
<td>ABCA1 KO</td>
<td>5'-TTTCTCATAGGGTTGCT CA-3'</td>
<td>5'-TGCAATCCATCTTGTTC AAT-3'</td>
<td>800</td>
</tr>
<tr>
<td>SR-BI</td>
<td>5'-GATGGGACATGGGGACA CGAAGCCATTCT-3'</td>
<td>5'-TCTGTCTCCGTCTCCTT CAGGTCTCTGA-3'</td>
<td>1000 for WT, 1200 for KO</td>
</tr>
</tbody>
</table>
### Supplementary Table 2: Hepatic mRNA expression levels measured by quantitative real-time PCR in different genotypic mice

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank Accession No.</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Amplicon Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCA1</td>
<td>NM013454</td>
<td>GGTGTTGGAGATGGTTATACCATAGCTG</td>
<td>TTCCCGAAACGCAAGTC</td>
<td>96</td>
</tr>
<tr>
<td>SR-BI</td>
<td>NM016741</td>
<td>GGCTGCTGTTTGGCTGCCG</td>
<td>GCTGCTGGATGAGGGAGGGA</td>
<td>63</td>
</tr>
<tr>
<td>LDLR</td>
<td>Z19521</td>
<td>CTGTGAGCTCCATAGGCTATC</td>
<td>GCCGCTCGAGGTCATCTC</td>
<td>68</td>
</tr>
<tr>
<td>LRP1</td>
<td>NM008512</td>
<td>TTGGGTCTCCCCGGAATCGTT</td>
<td>ACCACCCGATTCTTGAGGA</td>
<td>95</td>
</tr>
<tr>
<td>HPRT</td>
<td>J00423</td>
<td>TTGCTGAGATGTGCTGAAGG</td>
<td>AGCAGGTGAGCAAAGAACTTCTAGG</td>
<td>91</td>
</tr>
<tr>
<td>GAPDH</td>
<td>NM008084</td>
<td>TCCATGACAAACTTTGGCATTG</td>
<td>TCACGCCCACAGCTTTCCAGA</td>
<td>103</td>
</tr>
<tr>
<td>36B4</td>
<td>NM007475</td>
<td>GGACCCGGAAGAGACCTCTTT</td>
<td>GCACATCACCTCGAGAATTTCCTAG</td>
<td>85</td>
</tr>
<tr>
<td>β actin</td>
<td>X03672</td>
<td>AACCGTGAAAAAGATGACCCAGAT</td>
<td>CACAGCTGGATGGCTACAAGTACG</td>
<td>75</td>
</tr>
</tbody>
</table>

### Supplementary Table 3. Recovery of radioactivity in tissues in ABCA1/SR-BI dKO mice

<table>
<thead>
<tr>
<th>Organ</th>
<th>WT</th>
<th>ABCA1 KO</th>
<th>SR-BI KO</th>
<th>dKO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>39.7±1.30</td>
<td>12.4±1.4</td>
<td>64.6±1.9</td>
<td>51.4±1.1</td>
</tr>
<tr>
<td>Liver</td>
<td>26.5±1.5</td>
<td>46.0±3.1</td>
<td>3.5±0.6</td>
<td>3.1±0.3</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.22±0.02</td>
<td>0.85±0.26</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Pancreas</td>
<td>nd</td>
<td>0.02±0.01</td>
<td>nd</td>
<td>0.01±0.01</td>
</tr>
<tr>
<td>Kidney</td>
<td>nd</td>
<td>0.03±0.02</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Adrenal</td>
<td>0.59±0.06</td>
<td>3.48±0.11</td>
<td>0.07±0.01</td>
<td>0.09±0.01</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.15±0.09</td>
<td>0.29±0.10</td>
<td>0.35±0.14</td>
<td>0.42±0.19</td>
</tr>
<tr>
<td>Small intestine</td>
<td>nd</td>
<td>1.15±0.30</td>
<td>nd</td>
<td>0.09±0.09</td>
</tr>
<tr>
<td>Large intestine</td>
<td>0.04±0.02</td>
<td>0.69±0.11</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Bladder</td>
<td>0.02±0.01</td>
<td>0.03±0.01</td>
<td>nd</td>
<td>0.06±0.01</td>
</tr>
<tr>
<td>thymus</td>
<td>0.02±0.01</td>
<td>0.06±0.01</td>
<td>nd</td>
<td>0.06±0.01</td>
</tr>
<tr>
<td>Lung</td>
<td>nd</td>
<td>0.25±0.02</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Heart</td>
<td>0.13±0.03</td>
<td>0.13±0.01</td>
<td>0.28±0.03</td>
<td>0.29±0.03</td>
</tr>
<tr>
<td>fat</td>
<td>0.40±0.07</td>
<td>0.48±0.08</td>
<td>0.29±0.05</td>
<td>1.09±0.06</td>
</tr>
<tr>
<td>muscle</td>
<td>0.84±0.32</td>
<td>2.55±0.32</td>
<td>1.03±0.20</td>
<td>3.41±0.21</td>
</tr>
<tr>
<td>Bone</td>
<td>5.49±0.68</td>
<td>6.39±1.08</td>
<td>5.70±0.52</td>
<td>8.57±0.27</td>
</tr>
<tr>
<td>skin</td>
<td>4.42±0.39</td>
<td>3.96±0.67</td>
<td>6.40±0.25</td>
<td>8.78±0.19</td>
</tr>
<tr>
<td>Recovery</td>
<td>78.6%</td>
<td>78.7%</td>
<td>82.3%</td>
<td>77.4%</td>
</tr>
</tbody>
</table>

HDL labeled with [3H]CEt was injected into the tail vein of female different genotypic mice at the age of 12-16 weeks old. At 4h after injection, the animals were sacrificed, serum was collected and tissues were removed. Tissues were assayed for [3H] counts and a correction was made for the contribution of serum to the measured organ associated radioactivity (See Materials and methods). Values are presented as mean ± SEM of 3 female animals at the age of 12-16 weeks old in each group. n.d.: not detectable. Significant difference p<0.001 vs WT, SR-BI KO, and dKO; p<0.01 vs WT and ABCA1 KO; p<0.01 and p<0.001 vs WT and SR-BI KO mice.
### Supplementary Table 4. Plasma lipid levels of ABCA1/SR-BI dKO mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>WT</th>
<th>ABCA1 KO</th>
<th>SR-BI KO</th>
<th>dKO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free cholesterol (mg/dL)</td>
<td>14.7±0.9</td>
<td>3.6±0.3a</td>
<td>80.5±2.0a,c</td>
<td>3.6±0.2a,d</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>61.6±2.1</td>
<td>8.3±0.7a</td>
<td>123.7±5.7a,c</td>
<td>10.5±1.3a,d</td>
</tr>
<tr>
<td>Phospholipids (mg/dL)</td>
<td>136.9±3.5</td>
<td>46.3±4.8a</td>
<td>157.5±2.4a,c</td>
<td>47.5±2.0a,d</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>51.9±3.6</td>
<td>32.9±3.8a</td>
<td>63.7±4.0b,c</td>
<td>30.8±2.8a,d</td>
</tr>
</tbody>
</table>

Plasma was collected from mice (mixed male and female) at the age of 12-16 weeks old on chow after overnight fasting for lipid analysis. Values are presented as mean ± SE M for 8-13 animals in each group. Statistically significant difference ap<0.001 and bp<0.05 vs WT; cp<0.001 vs ABCA1 KO; dp<0.001 vs SR-BI KO.

### Supplementary Table 5. Blood cell count of ABCA1/SR-BI dKO mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>WT</th>
<th>ABCA1 KO</th>
<th>SR-BI KO</th>
<th>dKO</th>
</tr>
</thead>
<tbody>
<tr>
<td>White blood cells (x10^6/mL)</td>
<td>12.6±1.0</td>
<td>11.7±0.6</td>
<td>11.2±0.4</td>
<td>10.9±0.7</td>
</tr>
<tr>
<td>Lymphocytes (x10^6/mL)</td>
<td>10.6±0.8</td>
<td>10.4±0.8</td>
<td>9.4±0.3</td>
<td>9.5±0.7</td>
</tr>
<tr>
<td>Monocytes (x10^6/mL)</td>
<td>0.45±0.10</td>
<td>0.32±0.04</td>
<td>0.37±0.05</td>
<td>0.35±0.03</td>
</tr>
<tr>
<td>Granulocytes (x10^6/mL)</td>
<td>1.54±0.25</td>
<td>1.03±0.12</td>
<td>1.17±0.11</td>
<td>0.91±0.12</td>
</tr>
<tr>
<td>Red blood cells (x10^9/mL)</td>
<td>11.0±0.2</td>
<td>11.5±0.2</td>
<td>10.0±0.1a,c,d</td>
<td>10.8±0.3</td>
</tr>
<tr>
<td>Reticulocytes (x10^9/mL)</td>
<td>0.51±0.02</td>
<td>0.49±0.03</td>
<td>2.12±0.10b,c,e</td>
<td>0.50±0.05</td>
</tr>
<tr>
<td>Reticulocytes (%)</td>
<td>45.8±2.5</td>
<td>42.2±2.4</td>
<td>214.9±15.3b,c,e</td>
<td>46.3±4.6</td>
</tr>
</tbody>
</table>

Blood were drawn from the tail vein of mice (mixed male and female) at the age of 12-16 weeks old on chow without fasting for cell counting by Sysmex hematology analyzer. Values are presented as mean ± SEM for 6-8 animals in each group. Statistically significant difference ap<0.01 and bp<0.001 vs WT; cp<0.001 vs ABCA1 KO; dp<0.05 and ep<0.001 vs dKO.
**Supplementary Table 6.** Hepatic lipid content and biliary lipid and bile salt secretion in ABCA1/SR-BI dKO mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>WT</th>
<th>ABCA1 KO</th>
<th>SR-BI KO</th>
<th>dKO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatic total cholesterol (mg/g)</td>
<td>51.2±3.2</td>
<td>52.8±2.1</td>
<td>56.8±6.1</td>
<td>54.4±2.5</td>
</tr>
<tr>
<td>Hepatic Phospholipids (mg/g)</td>
<td>69.5±3.3</td>
<td>77.9±1.9</td>
<td>81.4±8.9</td>
<td>69.8±5.1</td>
</tr>
<tr>
<td>Hepatic triglycerides (mg/g)</td>
<td>64.3±5.6</td>
<td>51.7±2.1</td>
<td>68.7±7.5</td>
<td>67.3±10.5</td>
</tr>
<tr>
<td>Biliary cholesterol secretion (nmol/min/100 g BW)</td>
<td>0.92±0.15</td>
<td>0.93±0.38</td>
<td>0.79±0.04</td>
<td>0.86±0.18</td>
</tr>
<tr>
<td>Biliary phospholipid secretion (nmol/min/100 g BW)</td>
<td>26.5±3.7</td>
<td>21.4±6.9</td>
<td>24.6±1.5</td>
<td>17.0±3.3</td>
</tr>
<tr>
<td>Biliary bile salt secretion (nmol/min/100 g BW)</td>
<td>177.8±35.7</td>
<td>119.9±48.6</td>
<td>179.1±14.2</td>
<td>109.7±19.0</td>
</tr>
</tbody>
</table>

Values are presented as mean ± SEM for 5-6 male animals at the age of 12-16 weeks old on chow diet in each group. BW, body weight. No significant differences were found among different genotypic mice in hepatic lipid content and biliary lipid and bile salt secretion.

**Supplemental References**


