Enhanced Foam Cell Formation, Atherosclerotic Lesion Development, and Inflammation by Combined Deletion of ABCA1 and SR-BI in Bone Marrow–Derived Cells in LDL Receptor Knockout Mice on Western-Type Diet

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Rationale: Macrophages cannot limit the uptake of lipids and rely on cholesterol efflux mechanisms for maintaining cellular cholesterol homeostasis. Important mediators of macrophage cholesterol efflux are ATP-binding cassette transporter 1 (ABCA1), which mediates the efflux of cholesterol to lipid-poor apolipoprotein AI, and scavenger receptor class B type I (SR-BI), which promotes efflux to mature high-density lipoprotein. ABCA1 and SR-BI in bone marrow–derived cells in mice leads to impaired efflux to lipid-poor apoAI, suggesting that macrophages have additional pathways via which cellular cholesterol can be exported. In addition to ABCA1, macrophages also express the ABC half-transporter ABCG1. In contrast to ABCA1, ABCG1 facilitates cellular cholesterol efflux from macrophages to mature HDL but not to lipid-free apolipoprotein.

Objective: The aim of the present study was to increase the insight into the putative synergistic roles of ABCA1 and SR-BI in foam cell formation and atherosclerosis.

Methods and Results: Low-density lipoprotein receptor knockout (LDLr KO) mice were transplanted with bone marrow from ABCA1/SR-BI double knockout mice, the respective single knockouts, or wild-type littermates. Serum cholesterol levels were lower in ABCA1/SR-BI double knockout transplanted animals, as compared to the single knockout and wild-type transplanted animals on Western-type diet. Despite the lower serum cholesterol levels, massive foam cell formation was found in macrophages from spleen and the peritoneal cavity. Interestingly, ABCA1/SR-BI double knockout transplanted animals also showed a major increase in proinflammatory KC (murine interleukin-8) and interleukin-12p40 levels in the circulation. Furthermore, after 10 weeks of Western-type diet feeding, atherosclerotic lesion development in the aortic root was more extensive in the LDLr KO mice reconstituted with ABCA1/SR-BI double knockout bone marrow.

Conclusions: Deletion of ABCA1 and SR-BI in bone marrow–derived cells enhances in vivo macrophage foam cell formation and atherosclerotic lesion development in LDLr KO mice on Western diet, indicating that under high dietary lipid conditions, both macrophage ABCA1 and SR-BI contribute significantly to cholesterol homeostasis in the macrophage in vivo and are essential for reducing the risk for atherosclerosis. (Circ Res. 2010;107:e20-e31.)

Key Words: atherosclerosis • cholesterol • cytokines • macrophages • neutrophils

The hallmark of atherosclerotic lesion development is the accumulation of macrophage foam cells. Transporters implicated in cholesterol efflux from macrophages include the ATP-binding cassette (ABC) transporters ABCA1 and ABCG1, and scavenger receptor class B type I (SR-BI). ABCA1 is a full-size ABC-transporter that facilitates cholesterol efflux to lipid-poor apolipoprotein (apo)AI. Total-body ABCA1 knockout mice and Tangier disease patients with dysfunctional ABCA1 display a virtual absence of high-density lipoprotein (HDL), showing the essential role for ABCA1 in HDL metabolism. Targeted inactivation of ABCA1 in bone marrow–derived cells in mice leads to increased atherosclerotic lesion formation, whereas overexpression of ABCA1 inhibits the progression of atherosclerosis. Macrophages lacking ABCA1, however, still have substantial ability to efflux cholesterol to HDL despite impaired efflux to lipid-poor apoAI, suggesting that macrophages have additional pathways via which cellular cholesterol can be exported. In addition to ABCA1, macrophages also express the ABC half-transporter ABCG1. In contrast to ABCA1, ABCG1 facilitates cellular cholesterol efflux from macrophages to mature HDL but not to lipid-free apolipoprotein.
teins.\textsuperscript{7,8} Furthermore, HDL levels are not affected in genetically engineered mice lacking ABCG1.\textsuperscript{8} Disruption of ABCG1 specifically in macrophages has only a moderate effect on atherosclerotic lesion development.\textsuperscript{9–11} Combined deletion of ABCA1 and ABCG1 on macrophages, however, led to a major impairment of cholesterol mass efflux to apoAI and HDL and a dramatic accumulation of foam cells in tissues.\textsuperscript{12–14} Transplantation of ABCA1/ABCG1 double knockout (KO) bone marrow into low-density lipoprotein receptor KO (LDLR KO) mice led only to modest atherosclerosis when challenged with a Western-type diet (WTD), which was associated with markedly decreased plasma cholesterol levels.\textsuperscript{12} In heterozygous LDLr KO mice fed a high cholesterol/choleate diet, disruption of ABCA1 and ABCG1 in bone marrow–derived cells, however, did not affect serum cholesterol levels and consequently led to markedly increased atherosclerotic lesion development as compared to mice receiving single ABCA1 KO or ABCG1 KO bone marrow.\textsuperscript{14} These studies clearly illustrate the importance of studying the effects of combined deficiency of cholesterol transporters to establish the importance of a specific transporter for preventing foam cell formation and atherosclerotic lesion development in vivo.

In addition to ABCA1 and ABCG1, SR-BI has also been implicated in macrophage cholesterol efflux. SR-BI facilitates the transport of cholesterol from macrophages down a concentration gradient to mature HDL and mediates the selective uptake of cholesterol esters from HDL by the liver.\textsuperscript{2,3} Complete disruption of SR-BI function in mice is associated with the accumulation of abnormally large HDL particles in the circulation, reflecting impaired delivery of cholesteryl esters to the liver.\textsuperscript{3} Bone marrow–specific deletion of SR-BI did not affect serum HDL cholesterol levels and inhibited early atherosclerotic development,\textsuperscript{15} whereas the progression of advanced lesions was induced, \textsuperscript{15–17} indicating a unique dual role for macrophage SR-BI in the pathogenesis of atherosclerosis. Recent studies using macrophages from SR-BI KO mice and inhibitors of SR-BI– and ABCA1-mediated efflux showed that macrophage SR-BI does not promote cholesterol efflux from murine macrophages in culture.\textsuperscript{18} In addition, reverse transport of cholesterol from SR-BI KO macrophages to feces after transfer into wild-type (WT) C57BL/6 mice was not affected.\textsuperscript{19} Based on these studies, the role of macrophage SR-BI for cellular cholesterol efflux was considered to be limited. However, studies from Yancey et al demonstrated that macrophages with a combined deficiency of SR-BI and apoE display a reduced efflux capacity and accumulate free cholesterol in lysosomes.\textsuperscript{20} Moreover, recently Cuchel et al showed that free cholesterol mobilization in vivo in response to reconstituted HDL infusion is primarily mediated by SR-BI and not ABCA1 or ABCG1.\textsuperscript{21} In addition, overexpression of apoAI in heterozygous LDLr KO mice can protect against atherosclerosis in the absence of macrophage ABCG1 and ABCA1.\textsuperscript{22} Thus, SR-BI might be more important for controlling cellular cholesterol homeostasis in vivo than was initially anticipated.

In the present study, we investigated the putative synergistic effects of combined disruption of ABCA1 and SR-BI in bone marrow–derived cells and thus macrophages on lipoprotein metabolism and atherosclerosis. Our results indicate that both ABCA1 and SR-BI in macrophages have a significant protective role in foam cell formation and WTD-induced atherosclerotic lesion development in vivo.

### Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Briefly, bone marrow transplantations were performed with ABCA1/SR-BI double KO mice as donors and LDLr KO mice as recipients. Plasma lipids were determined by enzymatic colorimetric assays and cytokines in serum by using the mouse Bio-Plex suspension array (Bio-Rad, Sweden). For histological analysis, cryostat sections were routinely stained with oil red O. Peritoneal leukocytes were analyzed with a hematology cell analyzer. Atherosclerotic lesion areas in oil red O–stained cryostat sections of the aortic root and coronary arteries and en face lesions in the aortic arch and thoracic aorta were quantified using the Leica image analysis system. Macrophage-cholesterol efflux studies were performed using bone marrow–derived macrophages and thioglycollate-elicited macrophages.

### Results

#### Reduced Very-Low-Density Lipoprotein/LDL Cholesterol Levels by Combined Deletion of ABCA1 and SR-BI in Bone Marrow–Derived Cells

To study the effects of combined macrophage ABCA1 and SR-BI deficiency on lipoprotein metabolism and atherosclerosis in vivo, LDLr KO recipient mice were transplanted with bone marrow from WT, ABCA1 KO, SR-BI KO, or ABCA1/ SR-BI double KO littermates. Deletion of ABCA1, SR-BI, or both ABCA1 and SR-BI in bone marrow–derived cells did not affect total serum cholesterol concentrations at 8 weeks after transplantation when fed a regular chow diet (Figure 1A). Furthermore, no significant effects were observed of single ABCA1 or combined ABCA1/SR-BI deletion on the cholesterol lipoprotein distribution profile on chow diet (Figure 1B). Single SR-BI deletion resulted in a 1.7-fold (n = 10, P < 0.05) lower HDL cholesterol levels, whereas very-low-density lipoprotein (VLDL) and LDL cholesterol levels showed a tendency to increased values, but this failed to reach statistically significant levels.
bone marrow, a clear increase in VLDL and LDL levels was observed, although to a lesser extent as compared to the mice transplanted with WT or SR-BI KO bone marrow. The increase in VLDL and LDL in the ABCA1/SR-BI double KO transplanted animals, however, was largely attenuated as compared to the other 3 groups (Figure 1D).

To investigate the effects of combined deletion of ABCA1 and SR-BI in bone marrow–derived cells on cholesterol homeostasis, food intake, and cholesterol absorption by the animals was analyzed at 16 weeks after transplant after 8 weeks of WTD feeding. Food intake was reduced by 15% (P<0.01) in ABCA1/SR-BI double KO transplanted animals (2.16±0.13 g/d, n=6) as compared to WT transplanted mice (2.55±0.28 g/d, n=6). Moreover, the intestinal cholesterol absorption was mildly reduced by 27% (P<0.05, n=4) in ABCA1/SR-BI double KO transplanted animals as compared to controls, whereas triglyceride absorption was reduced by 43% (P<0.05, n=4). In addition, we tested whether combined ABCA1 and SR-BI deletion in bone marrow–derived cells affected VLDL synthesis by in vivo activation of lipolysis using Triton WR1339. The VLDL production rate was significantly lower in mice lacking ABCA1 and SR-BI in bone marrow–derived cells (1814±228 µg/mL per hour, n=3) as compared to control transplanted animals (3337±570 µg/mL per hour, n=3, P<0.01). In addition, a 1.8-fold reduction in hepatic HMG-CoA (hydroxymethylglutaryl-coenzyme A) reductase mRNA expression from 0.30±0.5 in WT to 0.17±0.2 in ABCA1/SR-BI double KO transplanted animals (n=4, P<0.05) was observed. Hepatic lipase mRNA expression in the liver was not affected (data not shown). In summary, reduced food intake, impaired intestinal lipid absorption, and reduced VLDL production by the liver will have contributed to the observed reduction in serum cholesterol levels in de ABCA1/SR-BI double KO transplanted animals.

Enhanced Atherosclerotic Lesion Development in the Aortic Root on Combined Deletion of ABCA1 and SR-BI in Bone Marrow–Derived Cells

Atherosclerotic lesion development was analyzed in cryostat sections of the aortic root after 10 weeks of WTD feeding (18 weeks after transplant). As anticipated, deletion of ABCA1 and/or SR-BI in bone marrow–derived cells resulted in total erasure of the respective proteins in the lesions of the transplanted mice (Figure 2A). Selective disruption of either ABCA1 or SR-BI in bone marrow–derived cells affected VLDL synthesis by in vivo inactivation of lipolysis using Triton WR1339. The VLDL production rate was significantly lower in mice lacking ABCA1 and SR-BI in bone marrow–derived cells (1814±228 µg/mL per hour, n=3) as compared to control transplanted animals (3337±570 µg/mL per hour, n=3, P<0.01). In addition, a 1.8-fold reduction in hepatic HMG-CoA (hydroxymethylglutaryl-coenzyme A) reductase mRNA expression from 0.30±0.5 in WT to 0.17±0.2 in ABCA1/SR-BI double KO transplanted animals (n=4, P<0.05) was observed. Hepatic lipase mRNA expression in the liver was not affected (data not shown). In summary, reduced food intake, impaired intestinal lipid absorption, and reduced VLDL production by the liver will have contributed to the observed reduction in serum cholesterol levels in de ABCA1/SR-BI double KO transplanted animals.

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metric analysis of the composition of the lesions showed that the macrophage content of the lesions of LDLr KO mice transplanted with ABCA1/SR-BI double KO bone marrow (31±4%, n=9) was significantly reduced as compared to WT, single ABCA1 KO, and single SR-BI KO transplanted animals (46±6% [n=11, P<0.05], 41±4% [n=8, P<0.01], and 49±5% [n=8, P<0.01], respectively). In addition, a significant increase in the necrotic core content of lesions of ABCA1/SR-BI double KO transplanted animals (21±2%, n=9) was observed as compared to WT, single ABCA1 KO, and single SR-BI KO transplanted animals (8±2% [n=10, P<0.01], 15±3% [n=8, P<0.05], and 10±2% [n=8, P<0.05], respectively). No effect was observed on the collagen content of the lesions. The decreased macrophage content and increased necrotic area in atherosclerotic lesions of the ABCA1/SR-BI double KO transplanted animals are consistent with the presence of more advanced lesions.

In addition to the aortic root, lesion development was also determined in the aortic arch, thoracic aorta, and right coronary artery after 10 weeks of WTD feeding (Online Figure I). In WT and SR-BI KO transplanted mice, 4.0±0.2% and 4.6±1.0%, respectively, of the aortic arch was covered with lesion. Single deletion of ABCA1 and combined deletion of ABCA1 and SR-BI in bone marrow–derived cells induced a similar 2.3-fold increase (P<0.01) in the vessel area covered by lesion. In the thoracic aorta, only 1.4±0.2% of the vessel was covered with lesion in WT transplanted mice. Deletion of SR-BI in bone marrow–derived cells resulted in a 3.5-fold decrease (P<0.05) in the area covered by lesion. Importantly, also in the thoracic aorta, no additional induction of

lesion development was observed on combined deletion of ABCA1 and SR-BI as compared to single deletion of ABCA1 (2.9±0.7% and 3.2±0.5%, respectively). The lesion size in the coronary artery was 3.6±0.3×10^5 and 3.6±0.4×10^5 µm^2 in mice transplanted with WT and SR-BI KO bone marrow, respectively, after 10 weeks of WTD feeding. Deletion of ABCA1 induced a 2.2-fold increase in coronary artery lesion size to 8.0±1.6×10^5 µm^2 (P<0.05), whereas lesions in ABCA1/SR-BI double KO transplanted animals were 9.0±1.5×10^5 µm^2 (P<0.05).

**Massive Foam Cell Formation in Spleen and Peritoneum by Combined Deletion of ABCA1 and SR-BI in Bone Marrow–Derived Cells**

To assess potential morphological changes associated with combined ABCA1 and SR-BI deficiency in bone marrow–derived cells outside the vasculature, a necropsy of the transplanted mice was performed. Because of the WTD feeding period, massive lipid accumulation was induced in livers of the transplanted animals (Figure 3). However, quantitative lipid analysis revealed no differences in lipid accumulation in the livers of the different groups of transplanted mice (data not shown). Microscopically, no heavily lipid-laden foam cells were evident in the liver. Furthermore, no foam cells were found in the lamina propria of the intestines, the largest reservoir of macrophages in the body, of either of the transplanted groups. The Peyer patches, lungs, and lymph nodes of the ABCA1/SR-BI double KO transplanted mice showed slightly enhanced lipid accumulation (Figure 3). Most striking differences, however, were observed on spleen morphology. No significant effect of leukocyte

![Figure 2. Atherosclerotic lesion development in the aortic root of LDLr KO mice reconstituted with WT, ABCA1 KO, SR-BI KO, and ABCA1/SR-BI double KO bone marrow. A, Expression of ABCA1 and SR-BI was immunofluorescently detected (red) in the aortic roots of WT, ABCA1 KO, SR-BI KO, or ABCA1/SR-BI double KO transplanted LDLr KO mice at 18 weeks posttransplantation including a 10-week WTD-feeding period. Nuclei were stained with DAPI (blue). Original magnification, 10×10. B, Photomicrographs showing representative oil red O-stained sections (original magnification, 10×5) and mean atherosclerotic lesion size in the aortic roots of mice transplanted with WT (n=20), ABCA1 KO (n=20), SR-BI KO (n=20), or ABCA1/SR-BI double knockout (n=21) bone marrow. Each symbol represents the mean lesion area in a single mouse. The horizontal line represents the mean of the group (WT: 348±30×10^3 µm^2; ABCA1 KO: 496±53×10^3 µm^2; SR-BI KO 533±27×10^3 µm^2; double KO: 693±41×10^3 µm^2). Statistically significant difference: **P<0.01, ***P<0.001.**
SR-BI deficiency was observed on spleen weight (3.7±0.4 mg/g body weight [n=9] as compared to 3.8±0.3 mg/g [n=9] for WT transplanted mice, P>0.05). ABCA1 and combined deletion of ABCA1 and SR-BI, however, induced a 1.6-fold (6.0±0.9 mg/g, n=9, P<0.05) and a 4.5-fold (17.1±3.1 mg/g, n=9, P<0.001) increase in spleen weight, respectively. Analysis of oil red O–stained sections of the spleen indicated that WT and SR-BI KO transplanted animals displayed only a few lipid-laden cells in the spleen (Figure 4A). Deletion of ABCA1 in bone marrow–derived cells resulted in slightly enhanced accumulation of lipid-laden cells. However, spleens of the mice transplanted with ABCA1/SR-BI double KO bone marrow displayed massive lipid loading. The lipid loading was especially evident in the red pulp of the spleen, constituting the reticuloendothelial system of the spleen, where it colocalized with macrophages (Figure 4B). Analyses of the lipid content of the spleens showed no significant effect of combined ABCA1 and SR-BI deletion in bone marrow on phospholipid, triglyceride, and free cholesterol concentrations. However, substantial effects were observed on cholesteryl ester accumulation. Single ABCA1 deletion induced a 3.7-fold (n=5, P<0.05) increase in the cholesteryl ester content of the spleen as compared to WT transplanted animals, whereas single SR-BI deficiency in bone marrow resulted in 3-fold (n=5) lower splenic cholesteryl ester levels, which failed to reach statistical significance (Figure 4C). Combined deletion of bone marrow ABCA1 and SR-BI, however, induced a dramatic 12.5-fold (n=5, P<0.05) increase in cholesteryl ester accumulation in spleens.

Interestingly, in addition to the observed increase in spleen size, more lipid-laden macrophages were also present in the peritoneum of mice reconstituted with ABCA1/SR-BI double KO bone marrow (22±1.2%, n=11, P<0.001), compared to the WT reconstituted animals (2.5±0.5%, n=12) (Figure 5). In the peritoneum, increased numbers lipid-laden cells were also observed in single ABCA1 KO transplanted animals (7.5±1.1%, n=8, P<0.01), but the effect was less severe as compared to combined deletion of ABCA1 and SR-BI. In addition, a tendency to reduced lipid loading was observed in peritoneal macrophages from mice reconstituted with SR-BI KO bone marrow (0.2±0.04%, n=9, P>0.05), in line with the dual function of SR-BI in macrophage foam cell formation and atherosclerotic lesion development.15 Quantitative analysis of the lipid content of the peritoneal leukocytes confirmed that combined deletion of ABCA1 and SR-BI resulted in a highly significant (P<0.001) increase in cholesterol ester accumulation in peritoneal macrophages as compared to single deletion of ABCA1 (WT: 11±5 [n=4]; SR-BI KO:10±7 [n=4]; ABCA1 KO:44±4 [n=4]; ABCA1/SR-BI double KO: 249±38 μg/mg [n=4]).

**Combined Deletion of ABCA1 and SR-BI Leads to Reduced Cholesterol Efflux Capacity From Macrophages to Both apoAI and HDL**

To gain more insight into the mechanism behind the massive lipid accumulation in peritoneal leukocytes and macrophages of the spleen, in vitro cholesterol efflux experiments were performed (Figure 6A). As shown previously,15 macrophage SR-BI deficiency resulted in 24% (n=8, P<0.01) lower cholesterol efflux to HDL. Combined deletion of ABCA1 and SR-BI resulted in a similar 20% (n=8, P<0.05) decrease in cholesterol efflux to HDL. In addition, macrophages lacking both ABCA1 and SR-BI showed an almost complete absence

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**Figure 3. Effect of combined deletion of ABCA1 and SR-BI in bone marrow–derived cells in LDLr KO mice on lipid accumulation in liver, lung, lymph node, small intestine, and Peyer patches. At 18 weeks after transplant, including 10 weeks of WTD feeding, indicated organs were isolated from LDLr KO mice transplanted with WT, ABCA1 KO, SR-BI KO, and ABCA1/SR-BI double KO bone marrow. Cryostat sections were stained with oil red O to visualize lipid accumulation. Original magnification, 5×10.**
Cholesterol efflux experiments were also performed using macrophages laden with acetylated LDL. Under these conditions, however, no additional effect of combined deletion of ABCA1 and SR-BI over single deletion of ABCA1 was observed on cholesterol mass and 3H-label efflux because of absence of SR-BI expression in all groups examined (Online Figure II).

**Increased Inflammation by Combined Deletion of ABCA1 and SR-BI in Bone Marrow–Derived Cells**

Studies in patients with Tangier disease have suggested a dual function for ABCA1 in both lipid metabolism and inflammation.\(^3\) Furthermore, we have shown that disruption of ABCA1 in bone marrow–derived cells results in an enhanced recruitment of leukocytes into peripheral tissues.\(^4\) In agreement with our earlier data, ABCA1 deficiency in bone marrow–derived cells resulted in a 2-fold (\(P<0.01\)) increase in the number of resident leukocytes in the peritoneal cavity (6.17±0.96×10\(^6\) cells [\(n=8\)] as compared to 3.15±0.33×10\(^6\) cells [\(n=12\)] for WT transplanted animals; Figure 7). No effect of single SR-BI deficiency was observed on peritoneal leukocyte counts (2.69±0.21×10\(^6\) cells). Interestingly, combined deletion of ABCA1 and SR-BI in bone marrow–derived cells resulted in a more dramatic increase in peritoneal leukocyte accumulation (8.79±0.71×10\(^6\) cells; \(n=11\), \(P<0.001\) as compared to WT and SR-BI KO transplanted animals and \(P<0.01\) as compared to ABCA1 KO reconstituted mice). As indicated in Figure 7, the increased peritoneal leukocyte counts in the single ABCA1 KO reconstituted animals were the result of a 1.6-fold increase (\(n=8\), \(P<0.05\)) in macrophage counts and a 3.2-fold increase (\(n=8\), \(P<0.01\)) in lymphocytes. In the ABCA1/SR-BI double KO transplanted animals a similar increase in macrophage counts was observed. The lymphocyte counts, however, were more dramatically increased (5.4-fold, \(n=11\), \(P<0.001\)). In addition, a striking accumulation of neutrophilic granulocytes was observed in the peritoneal cavity, accounting for 20% of the increase in the peritoneal leukocyte count in mice transplanted with ABCA1/SR-BI double KO bone marrow. Combined deletion of ABCA1 and SR-BI did not affect the peripheral WBC counts (5.6±0.6×10\(^9\) cells/L, \(n=12\) as compared to 5.8±0.8×10\(^9\) cells/L, \(n=11\) for WT transplanted animals), nor the percentile composition of the blood cells (data not shown). The increased neutrophil counts in the peritoneal cavity were thus primarily the result of increased recruitment of neutrophils. Because no thioglycollate or other additional trigger was used to elicit the recruitment of neutrophils, an endogenous trigger inside the peritoneal cavity most likely has caused the recruitment of the cells. It is interesting to speculate that this is the consequence of the extensive foam cell accumulation inside the peritoneal cavity. Next, live neutrophils inside the lesions were visualized using a naphthol AS-D chloroacetate esterase activity kit. Surprisingly, based on this esterase activity assay, no significantly higher neutrophil counts were observed within the atherosclerotic lesions of ABCA1/SR-BI double KO transplanted mice (49±15 [\(n=8\)] versus 39±6 for WT transplanted mice [\(n=10\)], \(P=0.51\)) or in the adventitia surrounding the lesion (134±18 [\(n=8\)] versus 93±14 [\(n=10\)] for WT transplanted mice).
mice, \(P=0.08\)). In addition to the esterase activity assay, an immunohistochemical staining for the neutrophil-specific marker Ly-6G was performed. Interestingly, using this staining, we did find a highly significant increase in the Ly-6G–positive area of lesions of ABCA1/SR-BI double KO (5.2±0.8% \(n=5\), \(P<0.0001\)) versus WT (0.2±0.04% \(n=5\)) transplanted animals. Ly-6G staining was primarily localized in acellular regions of the corners of the necrotic areas of the lesions, indicating the increased presence of residues of neutrophils that had infiltrated the lesion.

An inflammatory response involves a complex set of events that, in addition to rearrangements of innate immune cell populations, include changes in the cytokine profiles. Therefore, also the serum cytokine levels were examined in the different groups of transplanted animals using a Bio-Plex suspension array. On chow diet, no effect on cytokine levels was observed (data not shown). After 4 weeks of WTD feeding, however, a significant 1.8-fold increase (\(n=6\), \(P<0.01\)) in the proinflammatory cytokine KC (murine ortholog of interleukin [IL]-8) was observed in mice reconstituted with ABCA1/SR-BI double KO bone marrow, whereas no significant effect was observed on the other cytokines tested (IL-1\(\beta\), IL-6, IL-5, IL-10, IL-12, tumor necrosis factor [TNF]-\(\alpha\), or RANTES). After 10 weeks of WTD feeding, an even more dramatic 17-fold (\(n=5\), \(P<0.001\)) increase in KC levels was observed in the ABCA1/SR-BI double KO transplanted animals, and, under these conditions, IL-12p40 levels were also highly increased (11-fold, \(n=5\), \(P<0.001\); Figure 8). In addition, an increase in the antiinflammatory cytokines IL-5 and IL-10 was observed in the ABCA1/SR-BI double KO reconstituted animals. The levels of the antiinflammatory cytokines, however, remained substantially lower as compared to the proinflammatory cytokines (7.2±3.7 and 10.0±3.7 pg/mL for IL-5 and IL-10 as compared to 139±16...
Figure 7. Enhanced leukocyte accumulation in the peritoneal cavity of LDLr KO mice transplanted with ABCA1/SR-BI double KO bone marrow. At 18 weeks after transplant, including 10 weeks of WTD feeding, the peritoneal cavity of the LDLr KO mice transplanted with WT (open bar), ABCA1 KO (gray bar), SR-BI KO (dark gray bar), and ABCA1/SR-BI double KO (black bar) bone marrow was lavaged, and the collected peritoneal leukocytes were analyzed using an automated Sysmex XT-2000iV Veterinary Hematology analyzer for total cell (TOTAL), macrophage (MACRO), lymphocyte (LYMPHO), and neutrophil (NEUTRO) counts. Values are means±SEM (n=8 to 12). Statistically significant difference: *P<0.05; **P<0.01; ***P<0.001; ns indicates nonsignificant.

pg/mL and 858±192 pg/mL for KC and IL-12p40, respectively). The effects on the cytokine levels thus increase with a prolonged duration of the WTD feeding period.

No Increased Foam Cell Formation and Atherosclerosis in the Aortic Root as Result of Combined Deletion of ABCA1 and SR-BI in Bone Marrow–Derived Cells in LDLr KO Mice on Chow Diet

To investigate whether the WTD-induced increase in proinflammatory cytokines is required to induce massive foam cell formation and promote atherosclerotic lesion development in the aortic root on combined disruption of ABCA1 and SR-BI in bone marrow–derived cells of LDLr KO mice, a bone marrow transplantation experiment was performed and atherosclerotic lesion development was analyzed at 14 and 20 weeks after transplantation while maintaining the mice on regular chow diet. At 14 weeks, WT transplanted animals developed small foam cell–rich lesions with an average size of 85±8×10³ μm² (n=11). Consistent with our previously published proatherogenic role of SR-BI in small foam cell–rich lesions, disruption of SR-BI in bone marrow–derived cells in LDLr KO mice on chow diet resulted in slightly smaller atherosclerotic lesions (62±7×10³ μm², n=12, P<0.05). Deletion of ABCA1 led to a significant 1.5-fold increase in lesion size (127±18×10³ μm², n=11, P<0.05). In contrast to the result obtained in mice fed WTD, no added effect of combined deletion of ABCA1 and SR-BI in bone marrow–derived cells was found in LDLr KO mice on chow diet (140±22×10³ μm², n=11) as compared to single deletion of ABCA1. Also at 20 weeks after transplantation, no added effect of combined ABCA1 and SR-BI deletion was found on chow as compared to single deletion of ABCA1 (data not shown).

Under chow conditions, similarly increased foam cell accumulation was also observed in the peritoneal cavity of ABCA1 KO and ABCA1/SR-BI double KO transplanted animals (1.1±0.11% [n=11] and 1.3±0.22% [n=10], respectively, as compared to 0.15±0.02% [P<0.001, n=11] and 0.25±0.07% [n=11, P<0.001] for WT and SR-BI KO transplanted mice). Foam cell counts, however, were much less as compared to the levels in transplanted animals challenged with WTD. No effect on total peritoneal leukocyte counts was observed (data not shown) and neutrophil counts remained low (∼5% in all groups). Furthermore, no lipid accumulation was observed in the spleens nor a change in spleen size was observed of ABCA1/SR-BI double KO transplanted animals. The WTD challenge is thus essential to induce the enhanced foam cell formation, atherosclerotic lesion development, and inflammation in LDLr KO mice on combined deletion of ABCA1 and SR-BI in bone marrow–derived cells.

Discussion

In the present study, we show that specific disruption of both ABCA1 and SR-BI in bone marrow–derived cells of LDLr KO mice led to an added increase in atherosclerotic lesion development in the aortic root on challenge with WTD, compared to single ABCA1 KO or SR-BI KO reconstituted LDLr KO mice, despite lower serum cholesterol levels. Massive lipid accumulation was found in peritoneal macro-

Figure 8. Increased circulating cytokine levels in LDLr KO mice transplanted with ABCA1/SR-BI double KO bone marrow. Serum from the transplanted LDLr KO mice was collected at 18 weeks after transplantation including 10 weeks of WTD feeding. A Bio-Plex suspension array was used to measure 8 different cytokines, including IL-1β, IL-5, IL-6, KC (murine IL-8), IL-10, IL-12p40, TNF-α, and RANTES. The concentrations of IL-5, IL-6 (murine IL-8), IL-10, and IL-12p40 in WT (open bars), ABCA1 KO (gray bars), SR-BI KO (dark gray bars), and ABCA1/SR-BI double KO (black bars) transplanted mice are shown. No significant differences were observed in the concentrations of IL-1β, IL-6, TNF-α, or RANTES between the 4 groups of mice. Values are means±SEM (n=5 to 10). Statistically significant difference: *P<0.05; **P<0.01; ***P<0.001; ns indicates nonsignificant.
phages, as well as macrophages in the red pulp of the spleens of LDLr KO animals reconstituted with ABCA1/SR-BI double KO bone marrow. Furthermore, in addition to the lipid parameters, a significant increase in inflammation markers was noticed in the ABCA1/SR-BI double KO transplanted mice on WTD, which is expected to have contributed to the observed dramatic increase in lesion development. In line with these findings, no enhanced foam cell formation and atherosclerotic lesion development was observed on combined deletion of ABCA1 and SR-BI as compared to single deletion of ABCA1 in bone marrow–derived cells in LDLr KO mice fed regular chow diet that did not show enhanced inflammatory markers. It is important to note that this might also be the consequence of the less advanced stage of lesion development in the animals on chow. In our earlier published studies, we found that SR-BI in bone marrow–derived cells induces early lesion development, does not affect intermediate lesions, and protects against the development of advanced lesions in the aortic root of LDLr KO mice. Furthermore, the site examined for atherosclerosis determines the outcome, because no additional effect of combined deletion of ABCA1/ SR-BI over single ABCA1 deletion was observed in less advanced lesions in the aortic arch, thoracic aorta, and coronary arteries of LDLr KO mice challenged with WTD. SR-BI on bone marrow–derived endothelial progenitor cells is important for endothelial regeneration and HDL stimulates endothelial cell migration in an NO-independent manner via SR-BI. Although, previously, no site-specific atheroprotective effects of endothelial progenitor cells were reported, it cannot be excluded that deletion of SR-BI in bone marrow–derived cells might accelerate lesion development as result of site-specific effects on endothelial repair by bone marrow–derived endothelial progenitor cells.

The massive lipid accumulation in peritoneal leukocytes and the red pulp of spleens on combined deletion of ABCA1 and SR-BI in LDLr KO mice on WTD highlights the pivotal role of these transporters in cellular lipid homeostasis in vivo under high dietary lipid conditions. Furthermore, it is interesting to speculate that the presence of ABCG1 is not sufficient to compensate for the absence of these 2 cholesterol transporters under these conditions. ABCA1 stimulates the active transport of both cholesterol and phospholipids from the cell to lipid-poor apoAI, but only little to mature HDL. SR-BI and ABCG1, on the other hand, require a lipid-poor apoAI acceptor specificity between ABCG1 and SR-BI would suggest that SR-BI also cooperatively works with ABCA1 in cholesterol export. However, recent siRNA-mediated knockdown studies using the RAW macrophage cell line suggested that the interaction of lipid-free apoAI with ABCA1 generates a particle that interacts with ABCG1, but not with SR-BI. However, the observed massive in vivo lipid loading in peritoneal leukocytes and spleens, as well as the increased atherosclerotic lesion development in the aortic root of ABCA1/SR-BI double KO transplanted animals on WTD, indicate that combined deletion of ABCA1 and SR-BI in bone marrow–derived cells does have an added effect on foam cell formation in vivo. Cholesterol efflux studies showed that combined deletion of ABCA1 and SR-BI leads to a complete ablation of apoAI-induced cholesterol efflux, similar to that observed on single deletion of ABCA1. However, in addition, a moderate reduction in HDL cholesterol efflux could be observed. It is tempting to speculate that the increased foam cell formation observed in vivo is the result of the combined impairment of both apoAI and HDL-mediated efflux. However, because the effects observed on cholesterol efflux in vitro were relatively small, it cannot be excluded that in addition to effects on cholesterol efflux, other mechanisms also contribute to the increased foam cell formation observed in the ABCA1/SR-BI double KO transplanted animals. Remarkably, enhanced in vivo foam cell formation in the peritoneum and spleen was observed on combined deletion of ABCA1 and SR-BI in bone marrow–derived cells, despite the presence of ABCG1. On the other hand, in recent studies with ABCA1/ABCG1 double KO transplanted animals, we and others showed that combined deletion of ABCA1 and ABCG1 in bone marrow–derived cells also results in massive foam cell formation despite the presence of functional SR-BI. It can be anticipated that ABCG1 and SR-BI might have different roles in cholesterol homeostasis at different stages of macrophage foam cell formation. As shown previously, the role of macrophage SR-BI in atherosclerotic lesion development is dual: it accelerates early atherosclerotic development, while it slows down the progression of more advanced lesions. In agreement, in the present study, the percentage of peritoneal macrophages with a single SR-BI deletion that are transformed into foam cells appear to be reduced. SR-BI is a multifunctional receptor capable of binding a wide array of native and modified lipoproteins, as well as mediating the bidirectional flux of cholesterol. This leads to the unique function of macrophage SR-BI that it can facilitate initial lesion formation by inducing the uptake of proatherogenic lipoproteins by macrophages and inhibit more advanced lesion formation by promoting cholesterol efflux when foam cells are heavily loaded with cholesterol.

Alternatively, SR-BI and ABCG1 might efflux cholesterol from different specific cellular compartments or functionally distinct cellular pools of cholesterol. Wang et al showed that LDL cholesterol is preferentially effluxed to HDL, whereas cholesterol from modified acetylated LDL is primarily effluxed to lipid-poor apoAI in an ABCA1-dependent fashion. In agreement, SR-BI only mediated LDL-cholesterol efflux and not acetylated LDL cholesterol efflux. However, it is currently unknown whether SR-BI and ABCG1 mediate efflux from distinct or similar cellular cholesterol pools. On combined deletion of ABCA1 and SR-BI, massive foam cell formation was primarily observed in peritoneal leukocytes and macrophages of the red pulp in the spleen. Interestingly, combined deletion of ABCA1 and ABCG1 in bone marrow–derived cells of LDLr KO mice resulted not only in massive
oil red O–positive lipid staining in peritoneal leukocytes and macrophages of the red pulp of the spleen, but also in other tissues rich in macrophages, including the liver, lymph nodes, lamina propria of the intestine, and Peyer patches. Importantly, in contrast to ABCA1/SR-BI double KO transplanted LDLr KO mice, ABCA1/ABCG1 transplanted animals did display foam cell accumulation in macrophage-rich organs on a chow diet, although less extreme than when challenged with WTD (unpublished observation from our group, 2010). Thus, although the similarity in acceptor specificity of SR-BI and ABCG1 suggests possible redundancy of these transporters on macrophages for cholesterol efflux to HDL, the biological environment of the macrophage and possibly the availability of substrates also apparently influence the in vivo importance of SR-BI and ABCG1. Generation of ABCG1/SR-BI double KO mice, ABCA1/ABCG1/SR-BI triple KOs, and ABCA1/SR-BI double KO mice overexpressing ABCG1 is expected to shed further light on the (in)dependent roles of these cholesterol transporters in macrophage foam cell formation and atherosclerosis.

Atherosclerotic lesion development results from a combination of hyperlipidemia and an inflammatory response. In LDLr KO mice reconstituted with ABCA1/SR-BI double KO bone marrow, excessive atherosclerosis develops in the aortic root, despite largely reduced serum cholesterol levels. Interestingly, the proinflammatory cytokines KC (murine IL-8) and IL-12p40 were greatly elevated in LDLr KO mice transplanted with ABCA1/SR-BI double-deficient bone marrow. Both IL-12 and IL-8 are important proatherogenic cytokines. Daily administration of IL-12 promotes atherosclerosis in young apoe KO mice, whereas targeted deletion of IL-12 and vaccination against IL-12 attenuates atherosclerotic lesion development in murine models of atherosclerosis. IL-8 is a powerful, independent predictor of cardiovascular events. Furthermore, deletion of KC (murine IL-8) in LDLr KO mice attenuates atherosclerosis. Interestingly, IL-8 production is dose-dependently induced in human macrophage foam cells as a response to cholesterol loading with modified LDL, suggesting that the observed increase in KC (murine IL-8) levels are a direct effect of the massive foam cell formation in the ABCA1/SR-BI double KO transplanted animals. Excessive free cholesterol accumulation in macrophage foam cells induces cytokine secretion as a result of endoplasmic reticulum stress triggered by excess cholesterol in the endoplasmic reticulum. No effect of combined ABCA1/SR-BI deficiency was observed on free cholesterol loading. Therefore, it is unlikely that increased endoplasmic reticulum stress as a result of excess free cholesterol is the general cause for the increased cytokine production. Alternatively, KC (murine IL-8) secretion by macrophages could be induced as a result of increased accumulation of oxysterols, including 25-hydroxycholesterol, 7β-hydroxycholesterol, and 7-ketocholesterol. KC (murine IL-8) triggers monocyte arrest on early atherosclerotic endothelium and plays a central role in macrophage accumulation in established fatty streak lesions. Interestingly, IL-8 is also one of the most potent chemoattractants for neutrophils and the increased levels of KC (murine IL-8) in the ABCA1/SR-BI double KO transplanted animals clearly parallel the increased accumulation of neutrophils in the peritoneal cavity. Neutrophils are short-lived phagocytic cells that serve as essential early cellular effectors of innate immunity and constitute the “first line of defense.” The sequestration of neutrophils into the peritoneal cavity is thus most likely a protective response induced by the accumulation of heavily lipid-laden peritoneal macrophages in absence of both SR-BI and ABCA1. Interestingly, neutrophil activation is increased in patients with significant coronary stenosis, and enhanced neutrophil infiltration is observed in culprit lesions in acute coronary syndromes, suggesting an important role for neutrophils in atherosclerosis. In agreement, neutrophil depletion reduces atherosclerotic lesion development in apoE KO mice. Although a clear increase in neutrophil accumulation was observed in the peritoneal cavity in ABCA1/SR-BI double KO transplanted mice, within the atherosclerotic lesions and in the adventitia surrounding the lesion, no significant increase in the amount of neutrophils was observed based on esterase activity. Immunohistochemical staining for the neutrophil-specific marker Ly-6G did show a significant increase in the Ly-6G–positive area of lesions of ABCA1/SR-BI double KO versus WT transplanted animals. Ly-6G–positive staining was primarily localized in acellular regions of the corners of the necrotic areas of the lesions, suggesting the increased presence of residues of neutrophils that had infiltrated the lesion. Necrotic core formation during lesion development can in turn elicit an inflammatory response, which could further increase the recruitment of neutrophils. Analysis of the potential contribution of neutrophils locally in the arterial wall to the excessive atherosclerotic lesion development in mice with a combined deletion of ABCA1 and SR-BI in bone marrow–derived cells forms an interesting future challenge.

In addition to the observed increase in the proinflammatory cytokines KC (murine IL-8) and IL-12p40, we also observed an increase in the antiinflammatory cytokines IL-5 and IL-10, probably as a feedback reaction to control the inflammatory response. Interestingly, several lines of evidence suggest that pro- and antiinflammatory cytokines can affect the amount of circulating lipids. Previously, we have shown that IL-10 overexpression in LDLr KO mice results in a significant reduction in plasma cholesterol levels. Interestingly, the ABCA1/SR-BI double KO transplanted animals with the highest IL-10 concentrations also had the lowest serum cholesterol levels. Furthermore, chronic inflammation is associated with reduced serum cholesterol levels. Although the mechanism of the inflammation-induced reduction in serum cholesterol levels is still largely unclear, it might have contributed to the lower serum cholesterol levels observed in this study. A recent study by Lo et al showed that disruption of the production of the potent proinflammatory cytokines LIGHT and lymphotixin, leading to reduced T-cell numbers, reduces serum cholesterol levels, was associated with increased hepatic lipase levels. Combined disruption of ABCA1 and SR-BI in bone marrow–derived cells, however, did not affect T-cell counts nor hepatic lipase expression in the liver. In contrast, we showed that food intake, intestinal lipid absorption, and HMGCoA expression and VLDL production by the liver were reduced, all processes that are likely to have
contributed to the observed reduction in serum cholesterol levels in the ABCA1/SR-BI double KO transplanted animals.

In conclusion, despite lower serum cholesterol levels, combined deletion of ABCA1 and SR-BI in bone marrow–derived cells induces massive foam cell formation and promotes inflammation and atherosclerotic lesion development in LDLr KO mice challenged with a WTD, indicating that both macrophage ABCA1 and SR-BI contribute significantly to healthy cholesterol homeostasis in the macrophage in vivo and are essential for reducing the risk for atherosclerosis.

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Disclosures

None.

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38. Li Y, Schwabe RF, DeVries-Seimon T, Yao PM, Gerbod-Giannone MC, Banka CL, Solan J, Santoro D, Santoro D, Brand K, Curtiss LK. LDLr-deficient mice transplanted with bone marrow from mice lacking both ABCA1 and SR-BI also had major increases in inflammatory cytokines after 10 weeks of receiving a high fat/high cholesterol diet. What New Information Does This Article Contribute?

What Is Known?

- Cholesterol accumulation in macrophages promotes atherosclerotic lesion development.
- The ATP-binding cassette (ABC) transporter ABCA1 facilitates cholesterol efflux from macrophages to apolipoprotein AI and protects against atherosclerotic lesion development.
- Scavenger receptor class B type I (SR-BI) facilitates a bidirectional flux of cholesterol between cells and high-density lipoprotein (HDL) down a concentration gradient.
- Macrophage SR-BI has a dual role in atherosclerosis: it promotes early lesion development and has a protective function in advanced lesions.

What New Information Does This Article Contribute?

- Combined deletion of ABCA1 and SR-BI in bone marrow–derived cells, including macrophages, by means of bone marrow transplantation induces massive foam cell formation in peritoneal cavities and spleens of low-density lipoprotein receptor (LDLr)-deficient mice after 10 weeks of receiving a diet enriched with fat and cholesterol.

- Atherosclerotic lesion development in aortic roots is increased, despite lower serum cholesterol concentrations in LDLr-deficient mice transplanted with bone marrow from mice lacking both ABCA1 and SR-BI after 10 weeks of a high fat/high cholesterol diet.
- LDLr-deficient mice transplanted with bone marrow from mice lacking both ABCA1 and SR-BI also had major increases in inflammatory cytokines after 10 weeks of receiving a high fat/high cholesterol diet.

A hallmark of atherosclerotic lesion development is accumulation of macrophage foam cells. Macrophages are incapable of limiting lipid uptake and therefore rely on cholesterol efflux mechanisms for maintaining cellular cholesterol homeostasis. Important mediators implicated in macrophage cholesterol efflux include ABCA1 and SR-BI. Macrophage ABCA1 is generally accepted to play an important protective role in atherosclerotic lesion development by facilitating efflux of cellular cholesterol. A role of macrophage SR-BI, however, is under debate because disruption of SR-BI does not affect cholesterol efflux from murine macrophages in culture or after transfer into the peritoneal cavity of wild-type mice. In the present study, we investigated the effects of combined disruption of ABCA1 and SR-BI in bone marrow–derived cells on in vivo foam cell formation and atherosclerosis. Our results indicate that, despite inducing lower serum cholesterol concentrations, combined deletion of ABCA1 and SR-BI in bone marrow–derived cells induced massive foam cell formation and promoted inflammation and atherosclerotic lesion development in LDLr-deficient mice, challenged with a high fat/high cholesterol diet. Thus, both macrophage ABCA1 and SR-BI contribute significantly to maintenance of cholesterol homeostasis in macrophages in vivo and are essential for reducing atherosclerotic risk.
Enhanced Foam Cell Formation, Atherosclerotic Lesion Development, and Inflammation by Combined Deletion of ABCA1 and SR-BI in Bone Marrow–Derived Cells in LDL Receptor Knockout Mice on Western-Type Diet

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SUPPLEMENTAL MATERIAL

Detailed Methods

Mice
ABCA1 knockout (ABCA1 KO) mice were a kind gift of Dr. G. Chimini (14) and SR-BI knockout (SR-BI KO) mice were obtained from Dr. M. Krieger (21). The ABCA1 KO and SR-BI KO mice (both at least 8 generations backcrossed to the C57Bl/6 background) were cross-bred to generate double heterozygous offspring, which were subsequently intercrossed to obtain the ABCA1/SR-BI double knockout (double KO) mice, and single ABCA1 KO, SR-BI KO, and wildtype (WT) littermates. LDL receptor knock out (LDLr KO) mice were obtained from the Jackson Laboratory (Bar Harbor, USA). All mice were housed in a light and temperature controlled environment. Food and water were supplied ad libitum. Mice were maintained on regular chow (RM3, Special Diet Services, Whitham, UK), or were fed a Western-type diet, containing 15% (w/w) total fat and 0.25% (w/w) cholesterol (Diet W, Special Diet Services, Whitham, UK). Drinking water was supplied with antibiotics (83 mg/L ciprofloxacin and 67 mg/L polymyxin B sulphate) and 6.5 g/L sucrose. 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 the Ethics Committee for Animal Experiments of Leiden University.

Bone Marrow Transplantation
Female LDLr KO mice (n=20/group), age 11 weeks, were lethally irradiated with a single dose of 9 Gy (0.19 Gy/min, 200 kV, 4 mA), 1 day before transplantation. Bone marrow was harvested by flushing the femurs and tibias from male ABCA1/SR-BI double KO mice, single ABCA1 KO littermates, single SR-BI KO littermates or non transgenic (WT) littermates. Irradiated recipients received 5 x 10^6 bone marrow cells by intravenous injection into the tail vein.

Assessment of Chimerism
The reconstitution of the transplanted bone marrow was determined using PCR on genomic DNA from bone marrow. The wildtype ABCA1 gene was detected using a forward primer (5’-TgggAACTCCTgCTAAATT-3’) and a reverse primer (5’-CCATgTgggTgTAgACA-3’) resulting in a 751 bp PCR-fragment. The mutant ABCA1 gene was detected using a forward primer (5’-TTTCTCATAgggTTgTCA-3’) and a reverse primer (5’-TgCAATCCATCTTgTTCAAT-3’) resulting in a 540 bp PCR-fragment. The wildtype and mutant SR-BI gene were detected using a forward primer (5’-gATgAACTgAggACACgAggCATTcT-3’) and a reverse primer (5’-CTgCTCCgTCTCCCTTAggTCCCTgA-3’) resulting in a 1000 bp PCR-fragment for the wildtype allele and a 1500 bp PCR-fragment for the mutant allelle. Primers were obtained from Eurogentec (Liege, Belgium).

Lipid Analyses
After an overnight fasting-period, 100 µl of blood was drawn from the mice (n=10/group) by tail bleeding. Triglycerides in serum were determined using a standard enzymatic colorimetric assay (Roche Diagnostics, Mannheim, Germany). The concentrations of cholesterol in serum were determined by incubation with 0.025 U/mL cholesterol oxidase (Sigma) and 0.065 U/mL peroxidase 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-antipyrene, 1% polyoxyethylene-9-lauryl Ether, and 7.5%
methanol). Absorbance was read at 490 nm. The distribution of cholesterol over the different lipoproteins in serum was determined by fractionation of 30 µL serum of individual mice using a Superose 6 column (3.2 x 300 mm, Smart-system; Pharmacia, Uppsala, Sweden). Cholesterol content of the effluent was determined as indicated. Splenic free cholesterol, cholesterol ester and triglyceride content were determined as described above after extraction according to Bligh and Dyer (1) and dissolving the lipids in 2% Triton X-100. Phospholipid content of the spleens was determined using a standard enzymatic colorimetric assay (Wako chemicals GmbH, Neuss, Germany).

**Intestinal lipid absorption**

The intestinal absorption of cholesterol and triglycerides was determined by a dual isotope ratio method 16 weeks post-transplant after 8 weeks Western-type diet feeding. Briefly, mice were fasted overnight and lipoprotein lipase activity was inhibited by intravenous injection of 500 mg/kg Triton WR1339 as a 15 g/dL solution in 0.9% NaCl (Sigma) to inhibit the clearance of chylomicrons. Subsequently, mice were dosed intragastrically with a bolus of 200µl olive oil containing 1 g/L cholesterol, 2 µCi of [14C] cholesterol (Amersham) and 2 µCi of [3H] triolein (Amersham). Blood was collected at 1, 2, 3, and 4h after gavage and serum was isolated for scintillation counting to determine the 14C and 3H activity. The percentage of cholesterol and triglyceride absorption was expressed as percentage of administered dose absorbed.

**In vivo VLDL production**

VLDL production was measured 16 weeks post-transplant after 8 weeks Western-type diet feeding. Hereafter, mice were injected intravenously with 500 mg of Triton WR-1339 (Sigma) per kg body weight as described above after an overnight fast to inhibit plasma VLDL clearance. Mice were fasted throughout the experiment. Blood samples were taken at 0, 1, 2, 3, and 4 hours after Triton WR1339 injection. Plasma triglycerides were analysed enzymatically and were related to the body mass of the animals. The hepatic VLDL production rate was calculated from the slope of the curve and expressed as g/h/kg body weight.

**Analysis of gene expression by Real-Time Quantitative PCR**

Total RNA was extracted from livers of transplanted animals at 18 weeks post-transplant after 10 weeks Western-type diet feeding by the acid guanidium thiocyanate-phenol chloroform extraction method according to Chomczynski et al. (2). cDNA was synthesised from 0.5–1 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 were designed using Primer Express 1.5 Software with the manufacturer’s default settings (Applied Biosystems). For detection of hepatic lipase 5’-CAGCCTGGGAGCGCAC-3’ and 5’-CAATCTTGTCTTCCCCGTCCA-3’ were used as forward and reverse primers, respectively. 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 18Sr-RNA.

**Histological Analysis of the Atherosclerosis**

To analyze the development of atherosclerosis at the aortic root, the transplanted LDLr KO mice (n=20/group) were sacrificed at 18 weeks after bone marrow transplantation (age 29 weeks). All mice were fed the Western-type diet for 10 weeks before sacrifice.
The arterial tree was perfused in situ with PBS and the heart was excised and stored in 3.7% neutral-buffered formalin (Formal-fixx; Shandon Scientific Ltd., UK) until use. The hearts were bisected just below the atria, and the base of the hearts plus aortic roots were taken for analysis. The hearts were then sectioned perpendicular to the axis of the aorta, starting within the heart and working in the direction of the aortic arch as described by Paigen et al. (3). Once the aortic root was identified by the appearance of aortic valve leaflets, alternate 10-µm sections were taken and mounted on gelatinized slides and stained with oil-red-O. The atherosclerotic lesion area in the sections was quantified by using a Leica image analysis system, consisting of a Leica DMRE microscope coupled to a camera and Leica QWin Imaging software (Leica Ltd., Cambridge, UK). Mean lesion area was calculated (in µm²) from 10 sections, starting at the appearance of the tricuspid valves. Cryostat sections of the aortic root were stained immunofluorescently with primary antibodies specific for murine ABCA1 (Santa Cruz, Santa Cruz, USA) or SR-BI (Abcam, Cambridge, UK) to visualize the ABCA1 and/or SR-BI expression in the lesions. Photomicrographs of sections immunofluorescently labeled for ABCA1 or SR-BI were taken using a Bio-Rad Radiance 2100 MP confocal laser scanning system equipped with a Nikon Eclipse TE2000-U inverted fluorescence microscope.

For morphological analysis, sections were stained with Masson’s Trichrome Accustain according to manufacturer’s instructions (Sigma). Neutrophils were visualized using a naphthol AS-D chloroacetate esterase activity kit (Sigma-Aldrich, Zwijndrecht, The Netherlands) according to manufacturer’s instructions.

Where indicated atherosclerosis was also analysed in oil-red-O stained cross sections of the right coronary artery near the aortic root and en face in the aortic arch and thoracic aorta. All analyses were performed blinded.

Peritoneal leukocyte analysis
Upon sacrifice of the transplanted LDLr KO mice at 18 weeks after transplantation (including 10 weeks Western-type diet), the peritoneal cavity of the mice was lavaged with 10 ml cold PBS to collect peritoneal leukocytes for quantification of neutrophil, lymphocyte, and macrophage counts using an automated Sysmex XT-2000iV Veterinary Hematology analyzer (Sysmex Corporation, Kobe, Japan). The XT-2000iV employs a fluorescent flow cytometry method using a fluorescent dye staining cellular DNA and RNA and a semiconductor laser to detect forward-, side-scattered, and fluorescent light. Corresponding samples were cytospun for manual confirmation and stained with oil-red-O for detection of lipid accumulation.

Macrophage cholesterol efflux studies
Macrophage cholesterol efflux studies were performed using bone marrow-derived macrophages (BMDM) and thioglycollate-elicited peritoneal macrophages (PM) isolated from WT, SR-BI KO, ABCA1 KO, and SR-BI/ABCA1 dKO mice fed chow diet. To label PM with ³H-cholesterol the in vivo method was used that was previously developed in our lab (4), allowing the study of cholesterol efflux with minimal culture times. Briefly, PM were elicited by intraperitoneal injection with 1 ml 3% Brewer’s thioglycollate medium (Difco, Detroit, USA). After five days, the elicited PM were labelled in vivo by intraperitoneal injection of ³H-cholesterol. The injection sample was prepared by dissolving 6.25 µCi of ³H-cholesterol in 6.25 µL ethanol and subsequent addition of 500 µl PBS of 37°C. At 3.5 hours after injection, PM were harvested and seeded on 24-well plates at a density of 0.5 x 10⁶ cells in 500 µL Dulbecco’s modified Eagle’s medium (DMEM)/0.2% free fatty acid free BSA. After 1 hour, nonadherent cells were removed by
washing. The cholesterol mass of the cells was 60.4±3.0, 64.6±2.2, 60.3±2.7, and 60.3±1.7 µg/mg cell protein for WT, ABCA1 KO, SR-BI KO, and ABCA1/SR-BI double knockout macrophages, respectively.

For culture of BMDM, bone marrow cells were isolated from both femurs and tibias, plated, and differentiated in DMEM supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 20% L929 cell-conditioned media (as a source of M-CSF), and penicillin-streptomycin for 6 days. Subsequently, cells were loaded with cholesterol for 48 hours with acetylated LDL (50 µg/mL) and 0.5 µCi/mL 3H-cholesterol in DMEM/BSA. The loading medium was removed and the cells washed twice in PBS, then incubated overnight (16 hours) with DMEM/BSA, supplemented with 22-hydroxycholesterol (10 µmol/L) and 9-cis retinoic acid (1 µmol/L).

Cholesterol efflux from in vivo labeled PM and cholesterol-loaded BMDM was subsequently studied by incubation of the cells with DMEM/BSA alone, or supplemented with either 10 µg/ml apoAI (Calbiochem) or 10 or 50 µg/ml human HDL (isolated according to Redgrave et al. (5)). After a 24-hours efflux period, radioactivity in the cells and medium was determined by liquid scintillation counting. Efflux of 3H-cholesterol label is defined as (dpm medium/ dpm cell +dpm medium) x 100%. When indicated cell and media samples were also extracted and analyzed for free and esterified cholesterol mass by HLPC, as previously described (6). Cell proteins were measured using the BCA assay (Pierce). For mass analysis, HDL samples were separately analyzed to allow correction for HDL cholesterol present in relevant media samples. Mass cholesterol efflux is expressed as the percentage of total cell cholesterol present in the medium. Basal efflux to media (in the absence of added acceptors) has been subtracted from the data shown.

**Western blot analysis**

Macrophages (peritoneal and BMDM) were generated as described above and scraped in 50 mM Tris-HCl, 100 mM NaCl, and 0.5% Triton X-100 in the presence of protease inhibitors (Roche Molecular Biochemicals). The protein content was determined by the Bio-Rad protein assay. Aliquots of 20 µg (for BMDM) or 40 µg of protein (for peritoneal macrophages) were separated by 6% SDS-PAGE electrophoresis and transferred onto nitrocellulose membranes. Immunolabelling was performed using polyclonal antibodies directed against SR-BI (Santa Cruz Biotechnology Inc) or ABCG1 (Novus) or a murine monoclonal antibody against ABCA1 (AC-10, kindly provided by Dr M. R. Hayden (Vancouver, Canada)).

**Cytokine serum levels**

At 8 (chow diet), 12 weeks (4 weeks Western-type diet), and 18 weeks (10 weeks Western-type diet) post-transplant blood was collected from the transplanted animals. Serum was separated by centrifugation and stored at -80°C until analysis. The mouse Bio-Plex suspension assay (Bio-Rad Laboratories AB, Sundbyberg, Sweden) was used to measure 8 different cytokines: interleukin (IL) 1β, IL-5, IL-6, KC (murine ortholog of IL-8), IL-10, IL-12, tumor necrosis factor (TNF)-α, and RANTES (regulated on activation and normally T cell expressed and secreted). The assay was performed according to the protocol of the manufacturer. In brief, serum samples were thawed on ice and centrifuged at 4500 rpm for 3 min at 4°C. After this initial step, serum was incubated with microbeads labeled with specific antibodies to one of the indicated cytokines for 30 min. Samples were washed after the incubation and were then incubated with the detection antibody cocktail. This step was followed by another wash step, and the beads were incubated with streptavidin-phycocerythrin for 10 min, again washed, and the concentration of each cytokine was determined using the array reader.
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.

Detailed methods References
Supplementary Figure I. Atherosclerotic lesion quantification in the right coronary artery (A), the aortic arch (B), and the thoracic aorta (C) of LDLr KO mice, transplanted with WT, ABCA1 KO, SR-BI KO, or ABCA1/SR-BI double KO (dKO) bone marrow. Each symbol represents the mean lesion area in the coronary artery of single mouse, or the ratio of lesion area to surface area in the aortic arch and thoracic aorta of single mouse. The horizontal bar indicates the mean value for the group. Statistically significant difference *p<0.05, **p<0.01 and ***p<0.001.
Supplementary Figure II. Cholesterol mass (A) and $^3$H-label (B) efflux from acetylated LDL-loaded macrophages to apoAI (10 µg/mL) and HDL (10 µg/mL). (C) Quantification of ABCA1, SR-BI, and ABCG1 protein by Western blotting. Values are means±SEM. Statistically significant difference *p<0.05 and ***p<0.001. Note that no additional effect of combined deletion of ABCA1 and SR-BI over single deletion of ABCA1 was observed on cholesterol mass and $^3$H-label efflux due to absence of SR-BI expression under this condition where macrophages are loaded with acetylated LDL.