CHAPTER 6

Leukocyte ABC-transporter A1 and LDL receptor play independent roles in atherosclerosis: the potential contribution of T cells

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Abstract

Objective: The present study aims to investigate whether LDL receptor (LDLr) deficiency influences the atheroprotective effects of leukocyte ABC-transporter A1 (ABCA1) in vivo.

Methods and Results: LDLr-/- mice were transplanted with bone marrow from LDLr-/-/ABCA1-/- mice, LDLr+/+ /ABCA1-/- mice, and their respective controls. Plasma cholesterol levels were significantly reduced upon deletion of leukocyte ABCA1 after 8 weeks of Western-type diet feeding. Also, leukocyte ABCA1 deficiency led to increased monocytosis, recruitment of macrophages into peripheral tissues, and macrophage foam cell formation. None of these effects was influenced by the deletion of the leukocyte LDLr. Despite relatively low plasma cholesterol levels, animals transplanted with LDLr-/- /ABCA1-/- and LDLr+/+ /ABCA1-/- bone marrow developed 1.6-fold (p<0.01) and 1.4-fold (p<0.05) larger lesions, compared to their respective controls. Two-way ANOVA analysis also demonstrated that deletion of the leukocyte LDLr reduced lesion development (p<0.001). Interestingly, in LDLr-/- /ABCA1-/- transplanted mice, the reduced lesion size was associated with reduced lymphocytosis and recruitment of T cells into the adventitia of lesions.

Conclusions: Leukocyte LDLr deficiency does not affect the atheroprotective effects of leukocyte ABCA1. Furthermore, in addition to promoting macrophage cholesterol efflux, leukocyte ABCA1 also protects against atherosclerosis through inhibiting lymphocytosis and recruitment of T cells into the adventitia of lesions.
**Introduction**

Macrophage foam cell formation is a hallmark of atherosclerosis. Since macrophages cannot limit lipid uptake, cholesterol efflux is crucial for prevention of macrophage foam cell formation and atherosclerosis. ABC-transporter A1 (ABCA1) facilitates cholesterol efflux to lipid-free or lipid-poor apoAI [1]. Patients with Tangier disease, due to loss-of-function mutations in the ABCA1 gene, display macrophage foam cell accumulation in peripheral tissues and premature atherosclerosis [2]. Bone marrow transplantation (BMT) studies in LDL receptor knockout (LDLr−/−) mice indicate that leukocyte ABCA1 is atheroprotective. Leukocyte ABCA1 deficiency accelerates atherosclerosis [3], while overexpression of ABCA1 in leukocytes prevents the progression of atherosclerosis [4]. The atheroprotective effects of leukocyte ABCA1 have been primarily attributed to ABCA1 in macrophages. Mice that specifically lack ABCA1 in macrophages and neutrophils were generated in the group of Dr. Parks by crossing ABCA1 floxed mice with mice expressing Cre recombinase under the control of the macrophage/neutrophil-specific lysozyme M promoter [5]. Surprisingly, specific deletion of macrophage ABCA1 on LDLr−/− background does not promote atherosclerosis [6]. BMT studies in LDLr−/− mice were performed using bone marrow from single ABCA1−/− mice and thus, in contrast to the LDLr−/− mice with specific knockdown of macrophage ABCA1, the leukocytes in the BMT animals do express the LDLr. Interestingly, a direct link between macrophage LDLr and ABCA1 expression has been suggested. Macrophages lacking the LDLr display reduced expression of ABCA1 [7]. In these macrophages, oxysterols cannot inactivate sterol regulatory element-binding protein 1 (SREBP1), which suppresses ABCA1 expression via microRNA33 [8]. Thus, impaired induction of ABCA1 in macrophages lacking the LDLr in response to oxysterol loading might explain why no effect was observed of specific deletion of macrophage ABCA1 on atherosclerosis.

To test if absence or presence of the LDLr determines the effects of leukocyte ABCA1 deficiency on atherosclerosis, bone marrow from LDLr−/−/ABCA1−/−, LDLr+/+/ABCA1−/−, and their respective controls was transplanted into LDLr−/− mice. After 8 weeks recovery, the animals were fed Western-type diet (WTD) for an additional 8 weeks before determination of atherosclerotic lesion formation. Our results clearly demonstrate that leukocyte ABCA1 deficiency led to an increased susceptibility to atherosclerotic lesion development both in the absence and presence of the LDLr. Importantly, the present study also for the first time provides evidence that suppression of lymphocytosis and T-cell recruitment into the adventitia of lesions contributes to the atheroprotective function of leukocyte ABCA1.

**Methods**

For detailed methodology, please see the data supplement, available online at http://atvb.ahajournals.org. Briefly, bone marrow transplantations were performed with LDLr+/+/ABCA1−−, LDLr−/−/ABCA1−−, and respective control mice as donors and LDLr−/− mice as recipients. Plasma lipids were determined by enzymatic colorimetric assays. Atherosclerotic lesion area and lesion composition in cryostat sections of the aortic root were quantified using the Leica image analysis system. Peritoneal leukocytes and white blood cells were analyzed with hematology cell analyzer. Macrophages (CD11b−Ly6G+) in the spleen were quantified using flow cytometry. Macrophages in the liver and T cells in atherosclerotic lesions were detected by immunolabelling with anti-F4/80 and anti-CD3 antibody, respectively.
Results

Leukocyte ABCA1 deficiency inhibited the WTD-induced increase in plasma cholesterol independently of LDL receptor expression

To investigate the effects of combined deficiency of leukocyte ABCA1 and LDLr on foam cell formation and atherogenesis, bone marrow from LDLr+/+ABCA1−/−, LDLr−/−ABCA1−/−, LDLr+/+ABCA1+/+, and LDLr+/+ABCA1−/− mice was transplanted into LDLr−/− mice, which represent an established model for the development of atherosclerosis. Successful reconstitution of the recipient mice with donor bone marrow was confirmed by analysis of LDLr and ABCA1 transcripts in genomic DNA from bone marrow of transplanted mice as described previously [3, 9] (data not shown). Deletion of ABCA1, LDLr, or both ABCA1 and LDLr in bone marrow-derived cells did not affect plasma total cholesterol levels when fed regular chow diet (277±8, 263±7, 286±5, and 289±8 mg/dL for LDLr+/+ABCA1+/+, LDLr−/−ABCA1+/+, LDLr+/+ABCA1−/−, and LDLr−/−ABCA1+/+ transplanted animals, respectively), as shown in Figure 1A. At 8 weeks after BMT, the animals were switched from regular chow diet to WTD to induce atherosclerotic lesion formation. After 8 weeks on WTD, the total plasma cholesterol levels in both LDLr+/+ABCA1+/+ and LDLr−/−ABCA1+/+ transplanted mice increased approximately 4-fold (1108±72 mg/dL) and 3.6-fold (1017±63 mg/dL) (Figure 1A), respectively. In line with previous studies [10, 11], deletion of ABCA1 in bone marrow cells resulted in lower plasma cholesterol levels upon challenge with WTD (568±14, p<0.001 vs LDLr+/+ABCA1−/− and LDLr−/−ABCA1−/− transplanted mice). Like LDLr+/+ABCA1+/− transplanted animals, LDLr−/−ABCA1+/− transplanted animals showed lower plasma cholesterol levels (611±16, p<0.001 vs LDLr+/+ABCA1+/+ and LDLr−/−ABCA1+/+ transplanted mice), indicating that the effect of leukocyte ABCA1 on plasma cholesterol levels is independent of LDLr expression (Figure 1A). The lower levels of plasma cholesterol in the LDLr+/+ABCA1+/− and LDLr+/+ABCA1−/− transplanted animals were mainly due to reduced VLDL and LDL levels (Figure 1B).

![Figure 1. Plasma cholesterol levels on chow and Western-type diet (A) and lipoprotein cholesterol distribution profile on Western-type diet (B) in LDLr−/− mice reconstituted with LDLr+/+ABCA1+/+, LDLr+/+ABCA1−/−, LDLr−/−ABCA1+/+, and LDLr+/+ABCA1−/− bone marrow. Values are mean±SEM. Statistically significant difference **P<0.001 vs LDLr+/+ABCA1−/− group; ***P<0.001 vs LDLr−/−ABCA1+/+ group.](image-url)
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Leukocyte ABCA1 and LDLr deficiency independently affected atherosclerotic lesion development

The development of atherosclerotic lesions was analyzed at the aortic root. After 8 weeks on WTD, atherosclerotic lesion size in the aortic root was $475\pm73\times10^3 \mu m^2$ in LDLr$^{+/+}$/ABCA1$^{+/+}$ and $359\pm41\times10^3 \mu m^2$ in LDLr$^{-/-}$/ABCA1$^{+/+}$ transplanted mice (Figure 2A and 2B). In line with previous studies [3, 10, 11], despite the lower plasma cholesterol levels, selective disruption of ABCA1 in bone marrow-derived cells resulted in a 1.4-fold increase ($681\pm33\times10^3 \mu m^2$, p<0.05) in the mean atherosclerotic lesion size as compared to LDLr$^{+/+}$/ABCA1$^{+/+}$ transplanted mice. Notably, LDLr$^{-/-}$/ABCA1$^{-/-}$ transplanted mice also developed 1.6-fold ($563\pm41\times10^3 \mu m^2$, p<0.01) larger atherosclerotic lesions as compared to LDLr$^{-/-}$/ABCA1$^{+/+}$ transplanted mice, indicating that leukocyte ABCA1 could protect against atherosclerosis even in the absence of LDLr expression (Figure 2A). Interestingly, two-way ANOVA analysis demonstrated not only the atheroprotective effect of leukocyte ABCA1 (p<0.001) but also a pro-atherogenic effect of LDLr expression on leukocytes (p<0.001). No interaction between the effect of leukocyte ABCA1 and LDLr deletion on atherosclerosis was observed (p=0.93) (Figure 2B).

Quantitative morphological analysis of the atherosclerotic lesions showed that the macrophage content in the lesions was significantly reduced in LDLr$^{+/+}$/ABCA1$^{-/-}$ (19.6±2.5%, p<0.001) and LDLr$^{-/-}$/ABCA1$^{-/-}$ (14.6±3.3%, p<0.001) transplanted mice as compared to LDLr$^{+/+}$/ABCA1$^{+/+}$ (35.8±3.3%) and LDLr$^{-/-}$/ABCA1$^{+/+}$ (41.3±3.8%) transplanted mice, respectively (Figure 2C and 2D). As determined by Masson's Trichrome staining, ABCA1 deficiency and combined deficiency of ABCA1 and LDLr in bone marrow-derived cells led to a 2.8-fold (9.5±1.4%, P<0.001) and 4.6-fold (11.8±1.0%, p<0.001) increase in collagen deposition in lesions as compared to LDLr$^{+/+}$/ABCA1$^{+/+}$ (3.4±0.7%) and LDLr$^{-/-}$/ABCA1$^{+/+}$ (2.6±0.4%) transplanted animals, respectively (Figure 2C and 2D). Furthermore, an increased necrotic core content was also evident in lesions of LDLr$^{+/+}$/ABCA1$^{-/-}$ (4.5-fold, P<0.01 vs LDLr$^{+/+}$/ABCA1$^{+/+}$) and LDLr$^{-/-}$/ABCA1$^{-/-}$ (5.0-fold, P<0.001 vs LDLr$^{-/-}$/ABCA1$^{+/+}$) transplanted animals (Figure 2D). Taken together, leukocyte ABCA1 deficiency led to more advanced lesion formation even in the absence of LDLr expression.

Leukocyte ABCA1 deficiency impaired cellular cholesterol efflux in vitro and increased foam cell formation in vivo independently of LDLr expression

Next, we determined the combined effect of leukocyte ABCA1 and LDLr deficiency on cellular cholesterol efflux using bone marrow-derived macrophages. As shown in Figure 3, LDLr deficiency did not influence cholesterol efflux from macrophages to apoAI, while ABCA1 deficiency, as expected, diminished macrophage cholesterol efflux to apoAI in both the absence and presence of the LDLr (>90% decrease, p<0.001). In contrast, macrophage cholesterol efflux to HDL was not affected by deletion of ABCA1, LDLr, or both (Figure 3). Moreover, cholesterol loading with LDL (Figure 3A) or oxidized LDL (Figure 3B) did not influence the effect of macrophage ABCA1 deficiency on cholesterol efflux. In line with the cholesterol efflux data, foam cells within the peritoneal cavity of both LDLr$^{+/+}$/ABCA1$^{-/-}$ (11.9±1.0%, p<0.001) and LDLr$^{-/-}$/ABCA1$^{-/-}$ (11.1±0.7%, p<0.001) transplanted mice were similarly increased as compared to LDLr$^{+/+}$/ABCA1$^{+/+}$ (1.1±0.3%) and LDLr$^{-/-}$/ABCA1$^{+/+}$ (1.5±0.4%) transplanted mice (Figure 4A). This was also evident from peritoneal cells stained for neutral lipids with oil-red-O (Figure 4B). These data indicate that the essential role of macrophage ABCA1 in prevention from foam cell formation is independent of LDLr expression.
Figure 2. Atherosclerosis in the aortic root of LDLr⁻/⁻ mice reconstituted with LDLr⁺⁺/ABCA1⁺⁺, LDLr⁺⁺/ABCA1⁻⁻, LDLr⁻⁻/ABCA1⁺⁺, and LDLr⁻⁻/ABCA1⁻⁻ bone marrow at 8 weeks on WTD. A, Photomicrographs showing representative oil-red-O-stained sections (50x). B, Mean atherosclerotic lesion size in the aortic root of mice. Each symbol (Left panel) represents the mean lesion area in a single mouse. The horizontal line represents the mean of the group. Statistically significant difference *P<0.05, **P<0.01, ***P<0.001. Two-way ANOVA analysis (right panel) of the effect of ABCA1 and LDLr on mean atherosclerotic lesion size in the aortic root of mice. C, Photomicrographs showing the lesion compositions in the aortic root of mice. Sections of the aortic roots were stained with antibody against Moma-2 to visualize macrophages (100x). Morphological staining of atherosclerotic lesions in the aortic root with Masson’s Trichrome Accustain, which stains cytoplasm and muscle fiber red and collagen blue (100x). D, Bar graphs showing the quantification of macrophages, collagen, and necrotic core content of lesions in the aortic root of mice, transplanted with LDLr⁺⁺/ABCA1⁺⁺ (open bar), LDLr⁺⁺/ABCA1⁻⁻ (gray bar), LDLr⁻⁻/ABCA1⁺⁺ (dark gray bar), and LDLr⁻⁻/ABCA1⁻⁻ (black bar) bone marrow. Values represent the mean±SEM. Statistically significant difference **P<0.01 and ***P<0.001 vs LDLr⁺⁺/ABCA1⁺⁺ group; ##P<0.01 and ###P<0.001 vs LDLr⁻⁻/ABCA1⁻⁻ group.
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Figure 3. Cholesterol efflux from LDL-loaded (A) and oxidized LDL-loaded macrophages to apoAI and HDL. Bone marrow macrophages from LDLr+/+/ABCA1+/+ (open bar), LDLr+/+/ABCA1-/- (gray bar), LDLr-/-/ABCA1+/+ (dark gray bar), and LDLr-/-/ABCA1-/- (black bar) transplanted mice were labeled for cholesterol efflux as described in the materials and methods. Basal efflux to media (in the absence of added acceptors) has been subtracted from the data shown. Values are mean±SEM (n=3 mice/group). Statistically significant difference **P<0.01, ***P<0.001 vs LDLr+/+/ABCA1+/+ group; ##P<0.01, ###P<0.001 vs LDLr-/-/ABCA1+/+ group.

Figure 4. Macrophage foam cell formation in the peritoneal cavity of LDLr-/- mice reconstituted with LDLr+/+/ABCA1+/+ (open bar), LDLr+/+/ABCA1-/- (gray bar), LDLr-/-/ABCA1+/+ (dark gray bar), and LDLr-/-/ABCA1-/- (black bar) bone marrow at 8 weeks on WTD. A, Quantification of macrophage foam cells as percentage of the total amount of macrophages. Values are mean±SEM. Statistically significant difference ***P<0.001 vs LDLr+/+/ABCA1+/+ group; ###P<0.001 vs LDLr+/+/ABCA1-/- group. B, Photomicrographs of oil-red-O-stained cytospins of peritoneal cells.
The deletion of leukocyte LDLr reduced lymphocytosis and inhibited the recruitment of T cells into the adventitia underlying the lesions of animals transplanted with ABCA1 KO bone marrow.

Deletion of ABCA1 in bone marrow-derived cells has been shown to enhance leukocytosis [3]. In line, also in the current study, the concentration of monocytes in the blood was increased ~2-fold in LDLr+/+/ABCA1+/+ (0.50±0.10×10⁶/mL, p<0.05) as compared to LDLr+/+/ABCA1−/− (0.23±0.03×10⁶/mL) transplanted mice. A similar increase was observed in LDLr−/−/ABCA1+/+ (0.44±0.07×10⁶/mL, p<0.05) transplanted mice as compared to LDLr−/−/ABCA1−/− (0.22±0.04×10⁶/mL) (Figure 5A). Moreover, in line with our previous study [3], leukocyte ABCA1 deficiency also resulted in increased numbers of macrophages in the peritoneal cavity (1.7-fold, p<0.001 vs WT), the spleen (1.5-fold,
In contrast, leukocyte LDLr deficiency did not affect the recruitment of macrophages into the organs studied. Also, LDLr\textsuperscript{+/+}/ABCA1\textsuperscript{+/+} transplanted animals showed increased macrophage recruitment in the peritoneal cavity (1.7-fold, p<0.001), the spleen (1.8-fold, p<0.05), and liver (1.6-fold, p<0.001) as compared to LDLr\textsuperscript{+/+}/ABCA1\textsuperscript{++/+} transplanted animals (Figure 5B, C, and D). Taken together, the effects of leukocyte ABCA1 on monocytosis and recruitment of macrophages into peripheral tissues are independent of leukocyte LDLr expression.

As shown in Figure 5E, single deletion of either leukocyte ABCA1 or leukocyte LDLr did not significantly affect circulating granulocytes, as compared to LDLr\textsuperscript{+/+}/ABCA1\textsuperscript{+/+} transplanted animals. However, as compared to LDLr\textsuperscript{+/+}/ABCA1\textsuperscript{+/+} transplanted mice, both LDLr\textsuperscript{+/+}/ABCA1\textsuperscript{+/−} and LDLr\textsuperscript{+/+}/ABCA1\textsuperscript{−/+} transplanted animals displayed a similar 1.7-fold reduction (p<0.05) in the circulating levels of granulocytes. Strikingly, despite the lower amount of circulating granulocytes, combined deletion of leukocyte LDLr and ABCA1 resulted in enhanced recruitment of granulocytes into the peritoneal cavity (3.8-fold, p<0.05 vs LDLr\textsuperscript{+/+}/ABCA1\textsuperscript{+/−}), similar to the effect of single ABCA1 deficiency (3.0-fold, p<0.05 vs LDLr\textsuperscript{+/+}/ABCA1\textsuperscript{+/−}) (Figure 5F).

In addition to increased monocytosis, leukocyte ABCA1 also led to increased lymphocytosis as evidenced by increased levels of circulating lymphocytes (1.8-fold, p<0.05 vs WT) (Figure 5G). Although deletion of the leukocyte LDLr alone did not significantly affect lymphocyte counts in the circulation, combined deletion of leukocyte
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LDLr and ABCA1 resulted in a 1.6-fold reduction (p<0.01) as compared to single leukocyte ABCA1 deficiency. In the peritoneal cavity lymphocyte counts were increased in LDLr+/+/ABCA1+/− (2.9-fold, p<0.001 vs LDLr+/+/ABCA1+/+) transplanted mice and LDLr−/−/ABCA1−/− (2.0-fold, p<0.05 vs LDLr−/−/ABCA1+/+) transplanted animals. Of note, the counts in the peritoneal cavity of LDLr−/−/ABCA1−/− transplanted animals were 1.6-fold lower (p<0.001) as compared to LDLr+/+/ABCA1−/− transplanted animals (Figure 5H). Single deletion of the leukocyte LDLr did not affect the already low amount of peritoneal lymphocytes as compared to LDLr+/+/ABCA1−/− transplanted mice.

T lymphocytes are important players in the progression of atherosclerotic lesions [12]. We next analyzed the number of T cells in the lesions and underlying adventitia. As shown in Figure 6A, the number of T cells was limited inside the lesions, and no significant difference in the number of T cells was observed in the lesions of the different groups. In contrast, more T cells were visible in the underlying adventitia (Figure 6B). Leukocyte ABCA1 deficiency resulted in a 1.7-fold increase (p<0.001 vs LDLr+/+/ABCA1+/+) in the recruitment of T cells into the adventitia. Also, LDLr−/−/ABCA1−/− transplanted animals showed 1.5-fold (p<0.05) more T cells in the adventitia as compared to LDLr−/−/ABCA1+/+ transplanted animals. Deletion of the leukocyte LDLr resulted in 1.2-fold lower amount of T cells in the adventitia, which however failed to reach the statistical significance. Combined deletion of leukocyte LDLr and ABCA1 did lead to a significant reduction in recruitment of T cells into the adventitia (1.3-fold, p<0.01) as compared to single leukocyte ABCA1 deficiency. Of note, two-way ANOVA analysis demonstrated that leukocyte ABCA1 deficiency (p<0.001) increased, while leukocyte LDLr deficiency decreased (p<0.05) the recruitment of T cells into the adventitia of the atherosclerotic lesions. No interaction of leukocyte ABCA1 and LDLr on recruitment of T cells into the adventitia was observed (p=0.16) (Figure 6C). Interestingly, pearson correlation analyses demonstrated that the size of lesions was highly correlated with the number of T cells (r=0.72, p<0.0001) in the adventitia.

Discussion

Using bone marrow transplantation, we investigated the combined effects of leukocyte ABCA1 and LDLr deficiency on foam cell formation and atherosclerosis. In line with previous studies [3, 10, 11], transplantation of ABCA1−/−/LDLr+/− bone marrow into LDLr−/− mice led to increased foam cell formation and atherosclerosis. Importantly, ABCA1−/−/LDLr−/− transplanted animals also showed more foam cell formation and increased lesion development as compared to ABCA1+/−/LDLr−/− transplanted animals, clearly demonstrating that LDLr deficiency did not affect the atheroprotective function of leukocyte ABCA1. Interestingly, ABCA1+/−/LDLr+/− transplanted animals showed reduced lymphocytosis and less atherosclerotic lesion formation as compared to ABCA1−/−/LDLr+/− transplanted animals, indicating a pro-atherogenic role of LDLr on leukocytes.

Zhou et al demonstrated that deletion of the LDLr in peritoneal macrophages impairs sterol-induced ABCA1 expression and ABCA1-mediated cholesterol efflux [7]. However, in our studies, cholesterol efflux from bone marrow-derived macrophages lacking the LDLr to either apoAI or HDL was not affected. Importantly, in line with our cholesterol efflux studies, absence of the LDLr in bone marrow-derived cells also did not enhance foam cell formation upon WTD challenge in vivo. Interestingly, deletion of the LDLr in bone marrow-derived cells did lead to a small but significant reduction in atherosclerosis susceptibility in both the presence and absence of ABCA1. The transcription of the LDLr on macrophages is quickly downregulated after cholesterol loading [13], which leads to the previously published limited contribution of the
macrophage LDLr to foam cell formation and atherosclerosis in LDLr−/− mice upon transplantation with LDLr+/+ bone marrow [9, 14]. In agreement, we found no effect of leukocyte LDLr deletion on macrophage foam cell formation in vivo. In contrast, the presence of the LDLr on lymphocytes is crucial for the proliferation of lymphocytes [15]. In the present study, the circulating lymphocyte count was reduced in ABCA1−/−/LDLr−/− transplanted animals as compared to ABCA1−/−/LDLr+/+ transplanted mice. Therefore, we speculate that the effect of leukocyte LDLr deficiency on atherosclerosis in the present study might be rather due to its effect on the proliferation of lymphocytes, which becomes evident under conditions where lymphocyte counts are highly elevated due to the absence of ABCA1.

Hypercholesterolemia induces leukocytosis, in particular monocytosis [16] and neutrophilia [17], which promotes the development of atherosclerosis. The cholesterol efflux transporters ABCA1 and ABCG1 inhibit hypercholesterolemia-induced leukocytosis by suppressing bone marrow myeloid cell proliferation [18]. In line, increased leukocytosis and monocytosis was observed in ABCA1−/− transplanted LDLr−/− animals upon WTD challenge in previous [3] and present studies. Of note, the absence of LDLr did not influence the effects of leukocyte ABCA1 deficiency on monocytosis. In addition, in agreement with our previous study [3], also elevated circulating lymphocyte counts were observed in animals transplanted with ABCA1−/− bone marrow. Cholesterol accumulation in T lymphocytes is associated with increased proliferation [19]. Increased lymphocytosis in ABCA1−/− transplanted animals might thus be the consequence of the impaired cholesterol efflux from lymphocytes via ABCA1. Interestingly, blockade of cholesterol uptake via LDLr could reverse it, indicating the importance of the LDLr-mediated cholesterol uptake pathway for the induction of lymphocytosis.

Extensive evidence suggests an atheroprotective role for macrophage ABCA1. First, bone marrow transplantation studies showed that deficiency of leukocyte ABCA1 results in increased atherosclerotic lesion formation [3] while overexpression of leukocyte ABCA1 prevents the progression of atherosclerosis [4]. Second, ABCA1-mediated cholesterol efflux is crucial for the prevention of macrophage foam cell formation in vitro and in vivo [3, 10, 11]. Third, macrophage ABCA1 functions as an anti-inflammatory molecule [5, 20, 21]. Fourth, ABCA1 deficiency impairs the migration of macrophages [22], which might lead to the retention of macrophages in lesions. However, Brunham et al recently showed that macrophage-specific inactivation of ABCA1 on the LDLr KO background did not change the susceptibility to atherosclerosis [6]. The authors hypothesized that the failure to pick up the effect of macrophage ABCA1 on atherosclerosis might be due to the LDLr−/− background, as LDLr−/− macrophages were shown to have significantly less ABCA1 expression compared to wild-type macrophages [7]. In the present study, we excluded this possibility by clearly showing that absence of the LDLr did not affect the effects of leukocyte ABCA1 deficiency on macrophage foam cell formation in vivo nor atherosclerotic lesion development. Brunham et al also hypothesized that the enhanced lymphocytosis observed in ABCA1 KO transplanted animals might contribute to the increased lesion formation upon deletion of ABCA1 in bone marrow-derived cells of LDLr−/− mice. Interestingly, in the present study, we found that absence of the leukocyte LDLr did reduce the circulating lymphocyte counts and T cell recruitment into the adventitia of lesions in ABCA1−/− transplanted animals. Importantly, the number of T cells in the adventitia underlying lesions was highly associated with the lesion size. Our data thus indicate that enhanced lymphocytosis and T cell infiltration into the adventitia might contribute to the effects of leukocyte ABCA1 deficiency on atherosclerosis. Generation of T cell-specific ABCA1−/− mice is needed in the future to elucidate the extent of the atheroprotective effects of ABCA1 on T cells. Of note,
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despite that ABCA1<sup>-/-</sup>/LDLR<sup>-/-</sup> transplanted animals developed less lesions as compared to ABCA1<sup>+/+</sup>/LDLR<sup>-/+</sup> transplanted animals, lesions of both types of animals contained more necrotic core material. Therefore, the effects of macrophage ABCA1 on the vulnerability of lesions also warrants further investigation.

In conclusion, deletion of the leukocyte LDLr does not affect the atheroprotective effect of leukocyte ABCA1. Furthermore, in addition to promoting macrophage cholesterol efflux, leukocyte ABCA1 also protects against atherosclerosis probably through inhibiting lymphocytosis and recruitment of T cells into the adventitia of lesions.

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References


### Supplementary methods

#### Mice

LDLr<sup>−/−</sup> mice were obtained from the Jackson Laboratory (Bar Harbor, USA). ABCA1<sup>−/−</sup> mice, a kind gift of Dr. G. Chimini [1] were backcrossed to the C57Bl/6 background for at least 8 generations. LDLr<sup>−/−</sup>/ABCA1<sup>−/−</sup> offsprings, which were generated from cross-breeding, were intercrossed to obtain the LDLr<sup>−/−</sup>/ABCA1<sup>−/−</sup> mice, LDLr<sup>−/−</sup>/ABCA1<sup>−/+</sup>, LDLr<sup>−/+</sup>/ABCA1<sup>−/−</sup> and LDLr<sup>−/+</sup>/ABCA1<sup>−/+</sup> littermates. 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<sup>−/−</sup> mice were lethally irradiated with a single dose of 9 Gy (0.19 Gy/min, 200 kV, 4 mA) at the age of 12 weeks. Bone marrow was harvested by flushing the femurs and tibias from male LDLr<sup>−/−</sup>/ABCA1<sup>−/−</sup> mice, LDLr<sup>−/−</sup>/ABCA1<sup>−/+</sup>, LDLr<sup>−/+</sup>/ABCA1<sup>−/−</sup>, and LDLr<sup>−/+</sup>/ABCA1<sup>−/+</sup>
littermates. Irradiated recipients received 5 x 10^6 bone marrow cells by intravenous injection into the tail vein at 1 day after irradiation.

**Plasma cholesterol analyses**

After an overnight fasting-period, 100 μL of blood was drawn from the mice by tail bleeding. 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-antipyrine, 1% polyoxyethylene-9-laurylether, 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.

**Histological Analysis of the Aortic Root**

To analyze the development of atherosclerosis at the aortic root, the transplanted LDLr^-/-mice (n=13-16/group) were sacrificed at 16 weeks after bone marrow transplantation. All mice were fed the Western-type diet for 8 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 (Formalin-fix; 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. [2]. 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., UK). Mean lesion area was calculated (in μm²) from 10 sections, starting at the appearance of the tricuspid valves. Macrophages and T cells were detected by incubation of consecutive sections with α-MOMA-2 (Research diagnostics, USA) and α-CD3 (Neomarkers, USA), respectively. For morphological analysis, sections were stained with Masson’s Trichrome Accustain according to manufacturer’s instructions (Sigma). All analyses were performed blinded.

**White blood cells and peritoneal leukocyte analysis**

Upon sacrifice of the transplanted LDLr^-/- mice at 16 weeks after transplantation, the blood and peritoneal leukocytes were collected as before [3]. Lymphocyte, monocyte/macrophage, and granulocyte counts were quantified 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 cytopspun 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). For culture of BMDM, bone marrow cells were isolated from both femurs and tibias, plated, and differentiated in Dulbecco’s modified Eagle’s medium (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. BMDM were subsequently harvested and plated at 0.5 x 10^6 cells/well in 24-well plates in medium without L cell-conditioned medium. The next day, the cells were washed and incubated with either 100 μg/mL LDL or 20 μg/mL oxidized LDL and 0.5 μCi/mL 3H-cholesterol in DMEM/0.2% BSA for 24 hours at 37°C to load the cells with.
cholesterol. Cholesterol efflux from BMDM was subsequently studied by incubation of the cells with DMEM/0.2% free fatty acid-free BSA alone, or supplemented with either 10 µg/mL apoAI (Calbiochem) or 50 µg/mL human HDL. After a 24-hours efflux period, radioactivity in the cells and medium was determined by liquid scintillation counting. Cholesterol efflux is defined as \( \frac{dpm_{\text{medium}}}{dpm_{\text{cell}} + dpm_{\text{medium}}} \times 100\% \).

**Quantification of macrophages in the spleen and liver**

Splenocytes were isolated and stained with fluorochrome-conjugate combinations of the following monoclonal antibodies against CD11b and Ly6G from eBioscience at 4°C for 30 min in labeling buffer (1% mouse serum in PBS). Flow cytometry was performed with FacsCalibur and then analyzed with CellQuest software (Becton-Dickinson, San Jose), correcting for nonspecific staining with isotype antibody controls.

Cryostat sections of the liver (6 µm) were collected and immunolabeled with rat anti-mouse F4/80 (BMA Biomedicals) for detection of macrophages. The positive area in liver was quantified by using the Leica image analysis system.

**Statistical Analysis**

Statistical analysis was performed using ANOVA and the Student-Newman-Keuls post-test (GraphPad InStat and Prism software). A Pearson r test was used to perform correlation analysis. A level of p<0.05 was considered significant.

**Supplemental References**

