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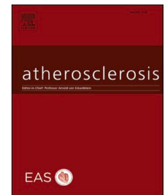

Impact of bone marrow ATP-binding cassette transporter A1 deficiency on atherogenesis is independent of the presence of the low-density lipoprotein receptor

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ABSTRACT

Background and aims: There is extensive evidence from bone marrow transplantation studies that hematopoietic ATP binding cassette A1 (Abca1) is atheroprotective in low-density lipoprotein receptor (Ldlr) deficient mice. In contrast, studies using lysosyme M promoter-driven deletion of *Abca1* in Ldlr deficient mice failed to show similar effects. It was hypothesized that the discrepancy between these studies might be due to the presence of Ldlr in bone marrow-derived cells in the transplantation model. In this study, we aim to determine the contribution of Ldlr to the atheroprotective effect of hematopoietic Abca1 in the murine bone marrow transplantation model.

Methods: Wild-type, $Ldlr^{-/-}$, $Abca1^{-/-}$, and $Abca1^{-/-}Ldlr^{-/-}$ bone marrow was transplanted into hypercholesterolemic $Ldlr^{-/-}$ mice.

Results: Bone marrow Lldr deficiency did not influence the effects of Abca1 on macrophage cholesterol efflux, foam cell formation, monocytosis or plasma cholesterol. Ldlr deficiency did reduce circulating and peritoneal lymphocyte counts, albeit only in animals lacking Abca1 in bone marrow-derived cells. Importantly, the effects of Abca1 deficiency on atherosclerosis susceptibility were unaltered by the presence or absence of Ldlr. Bone marrow Ldlr deficiency did lead to marginally but consistently decreased atherosclerosis, regardless of Abca1 deficiency. Thus, Ldlr expression on bone marrow-derived cells does, to a minimal extent, influence atherosclerotic lesion development, albeit independent of Abca1.

Conclusions: This study provides novel insight into the relative impact of Ldlr and Abca1 in bone marrow-derived cells on macrophage foam cell formation and atherosclerosis development *in vivo*. We have shown that Ldlr and Abca1 differentially and independently influence atherosclerosis development in a murine bone marrow transplantation model of atherosclerosis.

1. Introduction

The treatment and prevention of atherosclerosis are a major public health priority, as it represents the leading pathological contributor to cardiovascular disease. A hallmark of atherosclerosis is macrophage foam cell formation, i.e. excessive accumulation of cholesteryl esters in macrophages. Therefore, preventing macrophage foam cell formation is an important therapeutic strategy to lower atherosclerosis susceptibility, and understanding the mechanisms determining the balance between macrophage cholesterol uptake and efflux is essential to develop better treatment and prevention strategies.

Cholesterol efflux is an active mechanism, which is regulated by various transporters. Cholesterol efflux transporter ATP binding cassette A1 (Abca1) is present on the macrophage plasma membrane, and mediates cholesterol efflux to lipid poor apolipoproteins, such as Apolipoprotein (Apo) A1 and ApoE [1].

The importance of Abca1 in prevention of macrophage foam cell formation and atherosclerosis is evident from several bone marrow transplantation studies, in which Abca1 deficient or Abca1-overexpressing bone marrow was transplanted into

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hypercholesterolemic low-density lipoprotein receptor (Ldlr) deficient mice. First, Abca1 deficiency in bone marrow-derived cells results in increased atherosclerotic lesion formation [2], while overexpression of hematopoietic Abca1 prevents the progression of atherosclerosis [3]. Second, Abca1-mediated cholesterol efflux is crucial for the prevention of macrophage foam cell formation *in vitro* and *in vivo* [2,4,5]. Third, macrophage Abca1 functions as an anti-inflammatory protein [6–8]. Fourth, Abca1 deficiency impairs the migration of macrophages [9], which might lead to the retention of macrophages in lesions. However, studies using myeloid-specific lysosyme M promoter-driven deletion of *Abca1* in Ldlr deficient mice did not affect advanced stages of atherosclerotic lesion development, while only minor effects were observed on early lesions [10,11]. This triggered discussion about the role of macrophage Abca1 in the prevention of atherosclerosis.

There are several differences between bone marrow transplantation studies and lysosyme M promoter deletion studies that can explain the contradictory results. One major difference between the used experimental approach is the presence or absence of Ldlr in macrophages. In the bone marrow transplantation studies, which showed a strong atheroprotective effect of hematopoietic Abca1, Ldlr deficient mice were transplanted with bone marrow that was Abca1 deficient, but did express Ldlr. Thus, upon transplantation of the Abca1 deficient bone marrow, the expression of Ldlr in hematopoietic cells, such as macrophages and other leukocytes, is restored. This is in contrast to Ldlr deficient mice with lysosyme M promoter-driven Abca1 deficiency, in which leukocytes express neither Abca1 nor Ldlr.

However, effects of the reintroduction of Ldlr in bone marrow transplantation models are not yet fully understood. Although reconstitution of Ldlr in bone marrow-derived cells failed to reduce atherosclerosis susceptibility of Ldlr deficient mice [12,13], macrophage foam cell formation and atherosclerotic plaque size were significantly reduced in the absence of an effect on serum cholesterol levels in atherogenic diet-fed wild-type mice transplanted with Ldlr deficient bone marrow [14]. Interestingly, Ldlr deficient macrophages display significantly lower Abca1 levels compared to wild-type macrophages [15]. This opens up the possibility that the presence of Ldlr may be an important determinant for the role of Abca1 in foam cell formation and atherosclerosis prevention in our bone marrow transplantation model.

In this study, we therefore specifically examined the contribution of the presence of Ldlr on the atheroprotective effect of hematopoietic Abca1 in a murine bone marrow transplantation model for atherosclerosis.

2. Materials and methods

2.1. Mice

Ldlr deficient (*Ldlr*$^{-/-}$) mice were obtained from the Jackson Laboratory (Bar Habor, USA). Abca1 deficient (*Abca1*$^{-/-}$) mice, a kind gift of Dr. G. Chimini [16], were backcrossed to the C57Bl/6 background for at least 8 generations. *Abca1*$^{-/+}$*Ldlr*$^{-/+}$ bone marrow double heterozygous offspring, which were generated from cross-breeding, were intercrossed to obtain wild-type, *Ldlr*$^{-/-}$, *Abca1*$^{-/-}$, and *Abca1*$^{-/-}$*Ldlr*$^{-/-}$ bone marrow littermates, of which bone marrow was used for transplantation. All mice were housed in a light and temperature-controlled environment and were fed regular chow diet (RM3, Special Diet Services, Whitham, UK) and water ad libitum unless otherwise specified. All experimental protocols were approved by the Ethics Committee for Animal Experiments of Leiden University and were performed in accordance with national laws.

2.2. Bone marrow transplantation

Donor bone marrow of male wild-type, *Ldlr*$^{-/-}$, *Abca1*$^{-/-}$, and *Abca1*$^{-/-}$*Ldlr*$^{-/-}$ littermates was isolated by flushing the femurs and tibias with PBS. Recipient *Ldlr*$^{-/-}$ (12 weeks old) female mice (n = 15/group) were irradiated with a single dose of 9 Gy (0.19 Gy/min, 200 kV, 4 mA) total body radiation using a 225 Smart Röntgen source (YXLON international, Copenhagen, Denmark) one day before transplantation, and received a single cell suspension of 5×10^6 bone marrow cells in PBS by intravenous injection into the tail vein. Drinking water of the recipient mice was supplemented with antibiotics (83 mg/L ciprofloxacin and 67 mg/L polymixin B sulfate) and 6.5 g/L, starting 5 days before transplantation.

After transplantation, mice were fed a regular chow diet during the reconstitution phase of 8 weeks, after which mice were challenged with a Western-type diet (WTD); 15% (w/w) total fat and 0.25% (w/w) cholesterol (Diet W, Special Diet Services, Whitham, UK) for 8 weeks to induce atherosclerotic lesion development. At 16 weeks post transplantation, mice were anesthetized with a mix of xylazine (70 mg/kg), ketamine (350 mg/kg), and atropine (1.8 mg/kg) and sacrificed by orbital exsanguination and perfusion with PBS, after which the organs were collected for further analysis. Successful reconstitution of the recipient mice with donor bone marrow was confirmed by analysis of *Ldlr* and *Abca1* transcripts in genomic DNA of the bone marrow of transplanted mice as described previously [2,13].

2.3. 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 × 300 mm, Smart-system; Pharmacia, Uppsala, Sweden). Cholesterol content of the effluent was determined as indicated.

2.4. Histological analysis of the aortic root

Upon 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. [17]. 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 μm^2) from 10 sections, starting at the appearance of the tricuspid valves. For morphological analysis, sections were stained with Masson's Trichrome Accustain according to manufacturer's instructions (Sigma). Macrophages were detected by incubation of consecutive sections with α-MOMA-2 (Research diagnostics, USA). All analyses were performed blinded.

2.5. 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 [5]. 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 cytospun for manual confirmation and stained with oil-red-O for detection of lipid accumulation.

2.6. Macrophage cholesterol efflux studies

Human HDL (density 1.21 to 1.063 g/mL) and LDL (density 1.063 to 1.019 g/mL) was isolated from plasma of healthy subjects by ultracentrifugation in a KBr discontinuous gradient and dialysed against PBS/1 mM EDTA as described by Redgrave et al. [18]. For generation of oxidized-LDL (oxLDL), LDL was oxidatively modified by incubation of 200 μg/mL of LDL with 10 μM $CuSO_4$ at 37 °C for 20 h. Oxidation was terminated by dialysis against PBS containing 0.5 mM EDTA for at least 24 h. 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 0.5×10^6 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 human LDL or 20 μg/mL oxidized human LDL and 0.5 μCi/mL ^{3}H-cholesterol in DMEM/0.2% BSA for 24 h 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 isolated human HDL [18]. After a 24-h efflux period, radioactivity in the cells and medium was determined by liquid scintillation counting. Cholesterol efflux is defined as ($dpm_{medium}/dpm_{cell} + dpm_{medium}$) x 100%.

2.7. Statistical analysis

Statistical analysis was performed using Graphpad Prism software (San Diego CA, USA, http://www.graphpad.com). The significance of differences was calculated using two-way analysis of variance (ANOVA) with Tukey post-test. Probability values (*p*) lower than 0.05 were considered significant.

3. Results

To test if the presence of Ldlr is required for the atheroprotective effect of hematopoietic Abca1, we transplanted wild-type, *Ldlr*$^{-/-}$, *Abca1*$^{-/-}$, and *Abca1*$^{-/-}$*Ldlr*$^{-/-}$ bone marrow into *Ldlr*$^{-/-}$ mice and determined the effect on macrophage foam cell formation and atherosclerosis susceptibility.

Deletion of bone marrow *Abca1*, *Ldlr,* or both did not affect plasma total cholesterol levels when fed regular chow diet, as shown in Fig. 1A. However, upon challenge with a high fat/high cholesterol WTD to induce atherosclerotic lesion formation, the bone marrow genotype did cause differences in plasma cholesterol levels. Total plasma cholesterol levels in wild-type and *Ldlr*$^{-/-}$ bone marrow-transplanted mice increased strongly in response to WTD feeding (4-fold and 3.6-fold, respectively compared to chow-fed levels; Fig. 1A). Deletion of *Abca1* in bone marrow-derived cells resulted in an attenuated increase in plasma cholesterol upon challenge with WTD (2.2-fold compared to chow-fed levels). Mice reconstituted with *Abca1*$^{-/-}$*Ldlr*$^{-/-}$ bone marrow showed a comparable WTD-induced increase in cholesterol (2.1-fold compared to chow-fed levels), indicating that the effect of hematopoietic Abca1 on plasma cholesterol levels is independent of Ldlr expression in bone marrow-derived cells (Fig. 1A). In both *Abca1*$^{-/-}$ and *Abca1*$^{-/-}$*Ldlr*$^{-/-}$ transplanted mice, the lower levels of plasma cholesterol were attributable to reduced VLDL and LDL cholesterol (Fig. 1B).

We next determined the combined effect of hematopoietic Abca1 and Ldlr deficiency on peritoneal macrophage foam cell formation and cholesterol efflux. As shown in Fig. 2, Abca1 deficiency increased peritoneal foam cell numbers independent of Ldlr genotype (11- and 10-fold respectively for *Abca1*$^{-/-}$ and *Abca1*$^{-/-}$*Ldlr*$^{-/-}$; two-way ANOVA: $p < 0.001$ for Abca1 genotype; Fig. 2A and B). These data indicate that the essential role of macrophage Abca1 in the prevention from foam cell formation is independent of the presence of Ldlr. In line, Ldlr deficiency did not influence cholesterol efflux from macrophages to ApoA1, while Abca1 deficiency diminished macrophage cholesterol efflux to ApoA1 in both the absence and presence of Ldlr (>90% decrease, $p < 0.001$ compared to respective controls; two-way ANOVA: $p < 0.001$ for Abca1 genotype; Fig. 2C and D). In contrast, macrophage cholesterol efflux to HDL was not affected by deletion of either *Abca1*, *Ldlr*, or both (Fig. 2C and D). Moreover, cholesterol loading with LDL (Fig. 2C) or oxidized LDL (Fig. 2D) did not influence the effect of macrophage Abca1 deficiency on cholesterol efflux.

Monocytosis is a risk factor for atherosclerosis development [19,20]. Deletion of Abca1 in bone marrow-derived cells causes monocytosis and induces higher peritoneal macrophage counts [2]. Therefore, we investigated the combined effects of Abca1 and Ldlr on the numbers of circulating monocytes in blood and macrophages in the peritoneal cavity. Hematopoietic Abca1 deficiency increased the number of circulating monocytes independent of *Ldlr* genotype (2-fold; two-way ANOVA: $p < 0.01$ for *Abca1* genotype; Fig. 3A). In line, we also found that hematopoietic Abca1 deficiency led to higher peritoneal macrophage numbers independent of *Ldlr* genotype (1.7-fold; two-way ANOVA: $p < 0.001$ for *Abca1* genotype; Fig. 3B). In summary, the effects of hematopoietic Abca1 on monocytosis and recruitment of macrophages into peripheral tissues are independent of hematopoietic Ldlr expression.

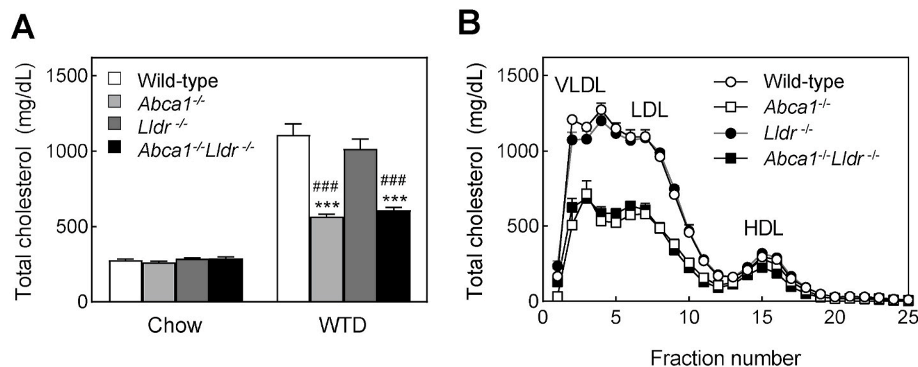

Fig. 1. Plasma cholesterol levels on chow and Western-type diet (A) and lipoprotein cholesterol distribution profile on Western-type diet (B) in *Ldlr*$^{-/-}$ mice reconstituted with wild-type, *Ldlr*$^{-/-}$, *Abca1*$^{-/-}$, and *Abca1*$^{-/-}$*Ldlr*$^{-/-}$ bone marrow.
WTD = Western-type diet. Values are mean ± SEM. Statistically significant difference $^{***}p < 0.001$ *vs* wild-type; $^{\#\#\#}p < 0.001$ *vs* *Ldlr*$^{-/-}$.

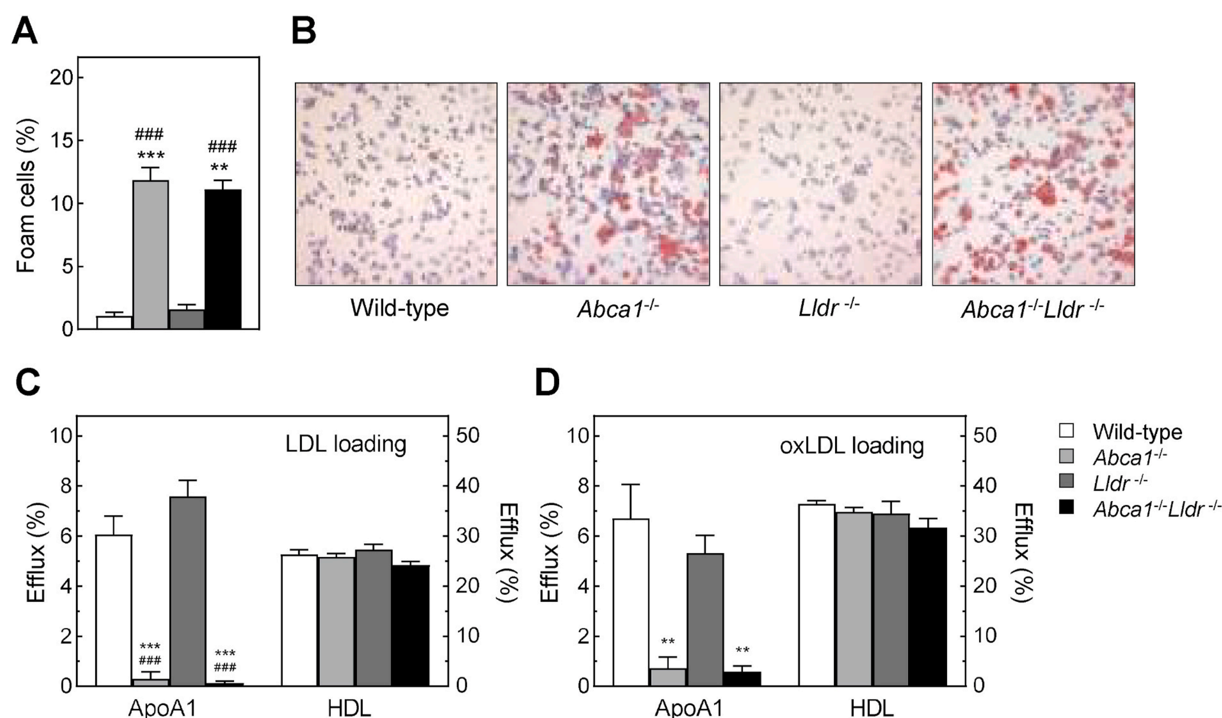

Fig. 2. Macrophage foam cell formation in the peritoneal cavity and cholesterol efflux of macrophages of *Ldlr*$^{-/-}$ mice reconstituted with wild-type, *Ldlr*$^{-/-}$, *Abca1*$^{-/-}$, and *Abca1*$^{-/-}$*Ldlr*$^{-/-}$ bone marrow at 8 weeks on Western-type diet.
(A) Quantification of macrophage foam cells as percentage of the total amount of macrophages. (B) Photomicrographs of oil-red-O-stained cytospins of peritoneal cells. (C and D) Bone marrow macrophages from *Ldlr*$^{-/-}$ mice reconstituted with wild-type, *Ldlr*$^{-/-}$, *Abca1*$^{-/-}$, and *Abca1*$^{-/-}$*Ldlr*$^{-/-}$ bone marrow transplanted mice were loaded with LDL (C) and oxidized LDL (D) 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* wild-type; $^{\#\#\#}p < 0.001$ *vs* *Ldlr*$^{-/-}$.

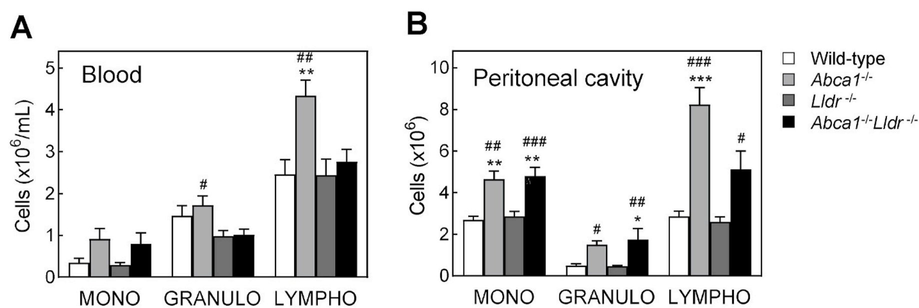

Fig. 3. Leukocytosis and recruitment of leukocytes into the peritoneal cavity in *Ldlr*$^{-/-}$ mice reconstituted with wild-type, *Ldlr*$^{-/-}$, *Abca1*$^{-/-}$, and *Abca1*$^{-/-}$*Ldlr*$^{-/-}$ bone marrow at 8 weeks on Western-type diet.
(A) Monocytes/macrophages (MONO), granulocytes (GRANULO), and lymphocytes (LYMPHO), in the blood. (B) Monocytes/macrophages (MONO), granulocytes (GRANULO), and lymphocytes (LYMPHO), in the peritoneal cavity. Values are mean ± SEM. Statistically significant difference $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs* wild-type; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *vs* *Ldlr*$^{-/-}$.

Circulating granulocytes were not affected by the presence of Abca1 in bone marrow-derived cells, but were decreased upon deletion of Ldlr (two-way ANOVA: not significant for *Abca1* genotype; $p < 0.01$ for *Ldlr* genotype; Fig. 3A). Interestingly, granulocyte counts in the peritoneal cavity were increased upon *Abca1* deletion in bone marrow-derived cells, but this effect was not influenced by the presence of Ldlr (two-way ANOVA: $p < 0.001$ for *Abca1* genotype; not significant for *Ldlr* genotype; Fig. 3B).

In addition to monocytes/macrophages and granulocytes, T lymphocytes also express both Abca1 [21] and Ldlr [22], and are of great importance in the progression of atherosclerotic lesions [23]. In addition to increased monocytosis, hematopoietic Abca1 deficiency also led to increased levels of circulating lymphocytes (1.8-fold, $p < 0.01$ *vs* wild-type) (Fig. 3A). Interestingly, although deletion of the hematopoietic *Ldlr* alone did not significantly affect lymphocyte counts in the circulation, combined deletion of hematopoietic *Ldlr* and *Abca1* reversed the effect of single hematopoietic Abca1 deficiency (1.6-fold reduction, $p < 0.05$ *vs* *Abca1*$^{-/-}$; Fig. 3B). In the peritoneal cavity, lymphocyte counts were increased in *Abca1*$^{-/-}$ bone marrow-transplanted mice (2.9-fold, $p < 0.001$ *vs* wild-type) and *Abca1*$^{-/-}$*Ldlr*$^{-/-}$ bone marrow-transplanted animals (2.0-fold, $p < 0.05$ *vs* *Ldlr*$^{-/-}$; Fig. 3B). Of note, the peritoneal lymphocyte counts of *Abca1*$^{-/-}$*Ldlr*$^{-/-}$ transplanted animals were 1.6-fold lower ($p < 0.01$) as compared to *Abca1*$^{-/-}$ bone marrow-transplanted animals. However, the *Abca1*$^{-/-}$ lymphocyte phenotype in the peritoneal cavity was not fully reversed by deletion of *Ldlr* in bone marrow-derived cells as observed in blood (Fig. 3B). Single deletion of the hematopoietic *Ldlr* did not affect peritoneal lymphocyte counts as compared to wild-type bone marrow-transplanted mice.

The development of atherosclerotic lesions was analyzed at the aortic root. After 8 weeks of WTD feeding, atherosclerotic lesion size in the aortic root was $475 \pm 73 \times 10^3$ μm^2 in wild-type and $359 \pm 41 \times 10^3$ μm^2 in *Ldlr*$^{-/-}$ bone marrow-transplanted mice (Fig. 4A). In line with previous studies [2,4,5], despite the lower plasma cholesterol levels, *Abca1*$^{-/-}$ bone marrow-transplantation resulted in a 1.4-fold increase ($681 \pm 33 \times 10^3$ μm^2, $p < 0.05$) in mean atherosclerotic lesion size as compared to wild-type bone marrow-transplanted mice. Notably,

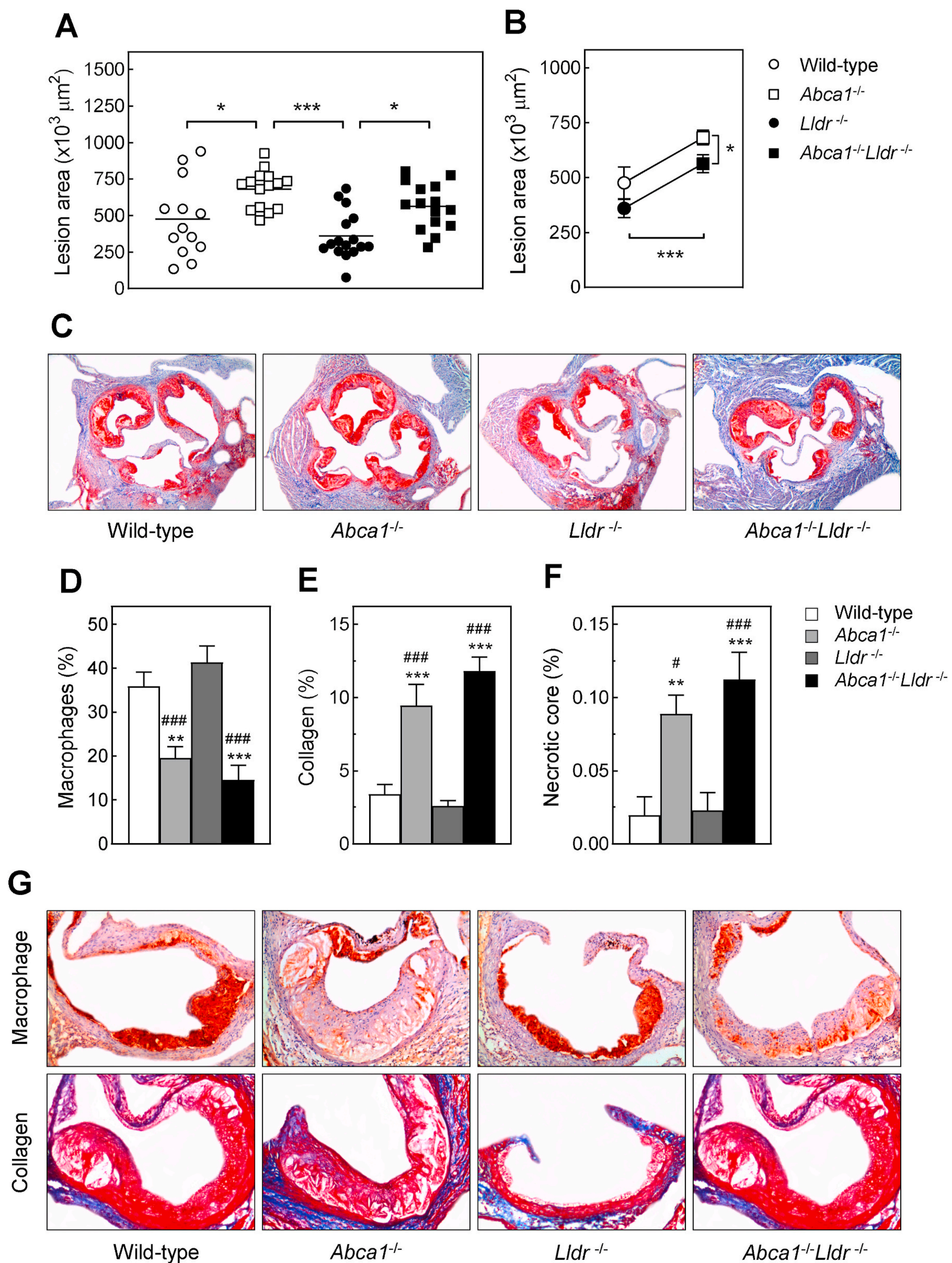

Fig. 4. Atherosclerosis in the aortic root in *Ldlr*$^{-/-}$ mice reconstituted with wild-type, *Ldlr*$^{-/-}$, *Abca1*$^{-/-}$, and *Abca1*$^{-/-}$*Ldlr*$^{-/-}$ bone marrow at 8 weeks on Western-type diet.
(A) Mean atherosclerotic lesion size in the aortic root. Each symbol represents the mean lesion area in a single mouse. The horizontal line represents the mean of the group. (B) Visualization of two-way ANOVA analysis of the effect of Abca1 and Ldlr on mean atherosclerotic lesion size in the aortic root of mice as represented in (A). (C) Photomicrographs showing representative oil-red-O-stained sections (50x). (D) Bar graphs showing the quantification of macrophages in the aortic root. (E) Bar graphs showing the quantification of collagen in the aortic root. (F) Bar graphs showing the quantification of necrotic core in the aortic root. (G) 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 cytoplasma and muscle fiber red and collagen blue (100x). Values are mean ± SEM. Statistically significant difference $^{**}p < 0.01$, $^{***}p < 0.001$ *vs* wild-type; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ *vs* *Ldlr*$^{-/-}$.

Abca1$^{-/-}$*Ldlr*$^{-/-}$ bone marrow-transplanted mice also developed 1.6-fold ($563 \pm 41 \times 10^3$ μm^2, $p < 0.05$) larger atherosclerotic lesions as compared to *Ldlr*$^{-/-}$ bone marrow-transplanted mice, indicating that the atheroprotective effects of hematopoietic Abca1 were preserved in the absence of Ldlr expression (Fig. 4A). Interestingly, two-way ANOVA analysis demonstrated not only the atheroprotective effect of hematopoietic Abca1 ($p < 0.001$) but also a small pro-atherogenic effect of Ldlr expression on hematopoietic cells ($p < 0.05$) (Fig. 4B), confirming the independent, opposite effects of Abca1 and Ldlr on atherosclerotic lesion size. Quantitative morphological analysis of the atherosclerotic lesions showed that, in line with the more advanced stage of lesion development, hematopoietic Abca1 deficiency decreases macrophage content in the lesions, while collagen content was increased, both independent of bone marrow *Ldlr* genotype (two-way ANOVA: $p < 0.001$ for *Abca1* genotype; Fig. 4D, E and G). Hematopoietic Abca1 deficiency also increased the necrotic core size in the lesions, independent of bone marrow *Ldlr* genotype (two-way ANOVA: $p < 0.001$ for *Abca1* genotype; Fig. 4F).

4. Discussion

There is extensive evidence from bone marrow transplantation studies from our group and others that hematopoietic Abca1 is atheroprotective. Deletion of *Abca1* in bone marrow-derived cells, including leukocytes, dramatically increases foam cell formation and strongly accelerates atherosclerosis in *Ldlr*$^{-/-}$ mice [2], while Abca1 overexpression in hematopoietic cells inhibits atherosclerosis progression [3]. Nonetheless, studies using lysosyme M promoter-driven deletion of *Abca1* in *Ldlr*$^{-/-}$ mice did not show increased atherosclerosis in advanced lesions, although some minor effects on early lesions were described [10,11]. The discrepancy between the observed atheroprotective effects in the different models may be due to the presence or absence of Ldlr. In the present study, we have determined the contribution of the presence of Ldlr on the atheroprotective effect of hematopoietic Abca1 in a bone marrow transplantation setting.

As shown previously [2,4,5], transplantation of *Abca1*$^{-/-}$ bone marrow into *Ldlr*$^{-/-}$ mice lowered macrophage cholesterol efflux and massively increased foam cell formation and monocytosis, which are both key determinants of atherosclerosis susceptibility. In accordance, the mean aortic root atherosclerotic lesion size was increased in mice transplanted with *Abca1*$^{-/-}$ bone marrow, despite lower cholesterol levels in these mice. Interestingly, the absence of bone marrow Ldlr did not influence the effect of Abca1 on macrophage cholesterol efflux, foam cell formation, monocytosis or plasma cholesterol. In line with these findings, the extent of the effects of Abca1 deficiency on foam cell formation and atherosclerotic lesion formation was also unaltered by the presence or absence of Ldlr. This clearly indicates that Ldlr does not influence the atheroprotective effect of hematopoietic Abca1 deficiency. Notably, since the consistently observed monocytosis in bone marrow transplantation studies with *Abca1*$^{-/-}$ bone marrow [2,4,5] is not present in the studies with lysosyme M promoter-driven deletion of Abca1 in the *Ldlr*$^{-/-}$ model [10,11], it is tempting to hypothesize that this may also provide an alternative explanation for the differential atheroprotective effects between the two models.

In addition to monocytosis, *Abca1*$^{-/-}$ bone marrow-transplanted animals display lymphocytosis [2]. This lymphocytosis possibly contributes to the increased lesion formation upon deletion of *Abca1* in bone marrow-derived cells of *Ldlr*$^{-/-}$ mice [10]. In the present study, we also observed Abca1 deficiency driven lymphocytosis after transplantation of *Abca1*$^{-/-}$ bone marrow into *Ldlr*$^{-/-}$ mice. However, interestingly, we found that the absence of hematopoietic Ldlr reduced circulating and peritoneal lymphocyte counts, albeit only in animals lacking Abca1 in bone marrow-derived cells. For circulating lymphocytes, the counts in *Abca1*$^{-/-}$*Ldlr*$^{-/-}$ bone marrow-transplanted mice were even normalized to numbers found in mice transplanted with wild-type or *Ldlr*$^{-/-}$ bone marrow. Cholesterol is crucial for cellular proliferation and therefore an important factor for rapid expansion of activated T cells. T cell activation increases expression of HMG-CoA reductase and Ldlr, and decreases the expression of Abca1 and Abcg1 to ensure adequate cholesterol availability for membrane biogenesis [21]. In accordance, cholesterol accumulation in T cells is associated with increased proliferation [24], and statin-induced reduction of de novo cholesterol synthesis inhibits T cell receptor driven expansion [25]. Moreover, liver X receptor (LXR) activation inhibits T cell expansion, whereas loss of LXRβ increases T cell expansion [21]. Increased lymphocytosis in *Abca1*$^{-/-}$ bone marrow transplanted animals might thus be the consequence of the impaired cholesterol efflux from lymphocytes via Abca1. Our findings suggest that lowered cholesterol uptake via Ldlr could possibly reverse this issue, which warrants future studies on the role of Ldlr-mediated cholesterol uptake pathway for the induction of lymphocytosis.

Another difference between bone marrow transplantation studies and studies using lysosyme M promoter-driven deletion can also be found in the difference in research methodology. Previous studies have shown that in bone marrow transplantation experiments, the lipid environment of the donor bone marrow still impacts bone marrow function after transplantation into recipients with a different lipid status. Effects of blood cholesterol levels and cholesterol efflux capacity on hematopoietic stem cells and hematopoiesis underly these long-term effects. Hypercholesterolemia and disruption of cholesterol efflux cause hematopoietic stem cell cholesterol accumulation, leading to monocytosis and granulocytosis [26,27]. In contrast, ApoA1 and HDL are able to suppress proliferation of HSCs and myeloid progenitor cells [27]. We and others have shown that hypercholesterolemia-induced hyperproliferation of HSCs, as well as the consequent leukocytosis, are also evident in recipients of bone marrow from hypercholesterolemic mice [28,29]. This opens up the possibility the bone marrow used for transplantation from different donors may have already been affected by plasma cholesterol levels in these donors. Bone marrow harvested from *Abca1*$^{-/-}$ mice, which are HDL deficient and hypocholesterolemic, may therefore already be different from bone marrow of wild-type or *Ldlr*$^{-/-}$ mice, which have normal or high blood cholesterol levels. This may partly account for differences in bone marrow transplantation studies compared to selective lysosyme M promoter-driven deletion. However, this remains speculation until future studies have shed light on the effects of low HDL levels on long term changes in bone marrow functioning.

Besides the primary aim to elucidate the influence of Ldlr on the atheroprotective function of hematopoietic Abca1, the study set up also allowed us to investigate the basic role of macrophage Ldlr in foam cell formation and atherosclerosis. Previous studies have shown that transcription of *Ldlr* on macrophages is quickly downregulated after cholesterol loading [30]. This limits the contribution of macrophage Ldlr to foam cell formation and atherosclerosis in wild-type bone marrow-transplanted *Ldlr*$^{-/-}$ mice [12,13]. In accordance, we found no effect of hematopoietic *Ldlr* deletion on macrophage foam cell formation *in vivo*. Previous bone marrow transplantation studies from our group and others, have yielded ambiguous results regarding the role of hematopoietic Ldlr on atherosclerosis. Attenuated atherosclerosis development was reported in wild-type mice transplanted with *Ldlr*$^{-/-}$ bone-marrow [12,14]. In contrast, in *Ldlr*$^{-/-}$ mice, reconstitution of the Ldlr in bone marrow-derived cells did not stimulate lesion development in two independent studies [12,13]. In the current study, due to the high statistical power (n = 13–16 mice/group and 4 separate groups), we did find marginally but consistently decreased atherosclerosis upon induction of Ldlr deficiency in bone marrow-derived cells. Thus, although Ldlr does not influence macrophage foam cell formation, Ldlr expression on bone marrow-derived cells does, to a minimal extend, influence atherosclerotic lesion development. More in-depth investigation is required to reveal the underlying mechanisms.

In conclusion, in this study we have provided novel insight into the relative impact of Ldlr and Abca1 on macrophage foam cell formation and atherosclerosis development in an *in vivo* bone marrow

transplantation model. We have shown that Ldlr and Abca1 differentially and independently influence atherosclerosis development. As such, Ldlr does not influence the atheroprotective effect of hematopoietic Abca1 in murine bone marrow transplantation models of atherosclerosis.

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CRediT authorship contribution statement

Amber B. Ouweneel: Formal analysis, Writing - original draft, Writing - review & editing, Visualization. **Ying Zhao:** Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization. **Laura Calpe-Berdiel:** Methodology, Investigation, Writing - review & editing. **Bart Lammers:** Methodology, Investigation, Writing - review & editing. **Menno Hoekstra:** Writing - original draft, Visualization, Writing - review & editing. **Theo J.C. Van Berkel:** Conceptualization, Methodology, Resources, Supervision, Writing - review & editing. **Miranda Van Eck:** Conceptualization, Methodology, Resources, Writing - original draft, Writing - review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

[1] N. Wang, D.L. Silver, P. Costet, A.R. Tall, Specific binding of ApoA-I, enhanced cholesterol efflux, and altered plasma membrane morphology in cells expressing ABC1, J. Biol. Chem. 275 (2000) 33053–33058, https://doi.org/10.1074/jbc.M005438200.

[2] M. van Eck, I.S.T. Bos, W.E. Kaminski, E. Orso, G. Rothe, J. Twisk, A. Bottcher, E. S. Van Amersfoort, T.A. Christiansen-Weber, W.-P. Fung-Leung, T.J.C. Van Berkel, G. Schmitz, Leukocyte ABCA1 controls susceptibility to atherosclerosis and macrophage recruitment into tissues, Proc. Natl. Acad. Sci. U.S.A. 99 (2002) 6298–6303, https://doi.org/10.1073/pnas.092327399.

[3] M. Van Eck, R.R. Singaraja, D. Ye, R.B. Hildebrand, E.R. James, M.R. Hayden, T.J. C. Van Berkel, Macrophage ATP-binding cassette transporter A1 overexpression inhibits atherosclerotic lesion progression in low-density lipoprotein receptor knockout mice, Arterioscler. Thromb. Vasc. Biol. 26 (2006) 929–934, https://doi.org/10.1161/01.ATV.0000208364.22732.16.

[4] R. Out, M. Hoekstra, K. Habets, I. Meurs, V. de Waard, R.B. Hildebrand, Y. Wang, G. Chimini, J. Kuiper, T.J.C. Van Berkel, M. Van Eck, Combined deletion of macrophage ABCA1 and ABCG1 leads to massive lipid accumulation in tissue macrophages and distinct atherosclerosis at relatively low plasma cholesterol levels, Arterioscler. Thromb. Vasc. Biol. 28 (2008) 258–264, https://doi.org/10.1161/ATVBAHA.107.156935.

[5] Y. Zhao, M. Pennings, R.B. Hildebrand, D. Ye, L. Calpe-Berdiel, R. Out, M. Kjerrulf, E. Hurt-Camejo, A.K. Groen, M. Hoekstra, W. Jessup, G. Chimini, T.J.C. Van Berkel, M. Van Eck, 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, Circ. Res. 107 (2010) e20–31, https://doi.org/10.1161/CIRCRESAHA.110.226282.

[6] X. Zhu, J.-Y. Lee, J.M. Timmins, J.M. Brown, E. Boudyguina, A. Mulya, A.K. Gebre, M.C. Willingham, E.M. Hiltbold, N. Mishra, N. Maeda, J.S. Parks, Increased cellular free cholesterol in macrophage-specific Abca1 knock-out mice enhances pro-inflammatory response of macrophages, J. Biol. Chem. 283 (2008) 22930–22941, https://doi.org/10.1074/jbc.M801408200.

[7] O.L. Francone, L. Royer, G. Boucher, M. Haghpassand, A. Freeman, D. Brees, R. J. Aiello, Increased cholesterol deposition, expression of scavenger receptors, and response to chemotactic factors in Abca1-deficient macrophages, Arterioscler. Thromb. Vasc. Biol. 25 (2005) 1198–1205, https://doi.org/10.1161/01.ATV.0000166522.69552.99.

[8] C. Tang, Y. Liu, P.S. Kessler, A.M. Vaughan, J.F. Oram, The macrophage cholesterol exporter ABCA1 functions as an anti-inflammatory receptor, J. Biol. Chem. 284 (2009) 32336–32343, https://doi.org/10.1074/jbc.M109.047472.

[9] M.P. Adorni, E. Favari, N. Ronda, A. Granata, S. Bellosta, L. Arnaboldi, A. Corsini, R. Gatti, F. Bernini, Free cholesterol alters macrophage morphology and mobility by an ABCA1 dependent mechanism, Atherosclerosis 215 (2011) 70–76, https://doi.org/10.1016/j.atherosclerosis.2010.12.004.

[10] L.R. Brunham, R.R. Singaraja, M. Duong, J.M. Timmins, C. Fievet, N. Bissada, M. H. Kang, A. Samra, J.-C. Fruchart, B. McManus, B. Staels, J.S. Parks, M.R. Hayden, Tissue-specific roles of ABCA1 influence susceptibility to atherosclerosis, Arterioscler. Thromb. Vasc. Biol. 29 (2009) 548–554, https://doi.org/10.1161/ATVBAHA.108.182303.

[11] X. Bi, X. Zhu, C. Gao, S. Shewale, Q. Cao, M. Liu, E. Boudyguina, A.K. Gebre, M. D. Wilson, A.L. Brown, J.S. Parks, Myeloid cell-specific ATP-binding cassette transporter A1 deletion has minimal impact on atherogenesis in atherogenic diet-fed low-density lipoprotein receptor knockout mice, Arterioscler. Thromb. Vasc. Biol. 34 (2014) 1888–1899, https://doi.org/10.1161/ATVBAHA.114.303791.

[12] M.F. Linton, V.R. Babaev, L.A. Gleaves, S. Fazio, A direct role for the macrophage low density lipoprotein receptor in atherosclerotic lesion formation, J. Biol. Chem. 274 (1999) 19204–19210, https://doi.org/10.1074/jbc.274.27.19204.

[13] N. Herijgers, M. Van Eck, P.H. Groot, P.M. Hoogerbrugge, T.J. Van Berkel, Effect of bone marrow transplantation on lipoprotein metabolism and atherosclerosis in LDL receptor-knockout mice, Arterioscler. Thromb. Vasc. Biol. 17 (1997) 1995–2003, https://doi.org/10.1161/01.atv.17.10.1995.

[14] N. Herijgers, M. Van Eck, P.H. Groot, P.M. Hoogerbrugge, T.J. Van Berkel, Low density lipoprotein receptor of macrophages facilitates atherosclerotic lesion formation in C57Bl/6 mice, Arterioscler. Thromb. Vasc. Biol. 20 (2000) 1961–1967, https://doi.org/10.1161/01.atv.20.8.1961.

[15] X. Zhou, W. He, Z. Huang, A.M.J. Gotto, D.P. Hajjar, J. Han, Genetic deletion of low density lipoprotein receptor impairs sterol-induced mouse macrophage ABCA1 expression. A new SREBP1-dependent mechanism, J. Biol. Chem. 283 (2008) 2129–2138, https://doi.org/10.1074/jbc.M706636200.

[16] J. McNeish, R.J. Aiello, D. Guyot, T. Turi, C. Gabel, C. Aldinger, K.L. Hoppe, M. L. Roach, L.J. Royer, J. de Wet, C. Broccardo, G. Chimini, O.L. Francone, High density lipoprotein deficiency and foam cell accumulation in mice with targeted disruption of ATP-binding cassette transporter-1, Proc. Natl. Acad. Sci. U.S.A. 97 (2000) 4245–4250.

[17] B. Paigen, A. Morrow, P.A. Holmes, D. Mitchell, R.A. Williams, Quantitative assessment of atherosclerotic lesions in mice, Atherosclerosis 68 (1987) 231–240.

[18] T.G. Redgrave, D.C. Roberts, C.E. West, Separation of plasma lipoproteins by density-gradient ultracentrifugation, Anal. Biochem. 65 (1975) 42–49, https://doi.org/10.1016/0003-2697(75)90488-1.

[19] C.D. Lee, A.R. Folsom, F.J. Nieto, L.E. Chambless, E. Shahar, D.A. Wolfe, White blood cell count and incidence of coronary heart disease and ischemic stroke and mortality from cardiovascular disease in African-American and White men and women: atherosclerosis risk in communities study, Am. J. Epidemiol. 154 (2001) 758–764.

[20] P.M. Sweetnam, H.F. Thomas, J.W. Yarnell, I.A. Baker, P.C. Elwood, Total and differential leukocyte counts as predictors of ischemic heart disease: the Caerphilly and Speedwell studies, Am. J. Epidemiol. 145 (1997) 416–421.

[21] S.J. Bensinger, M.N. Bradley, S.B. Joseph, N. Zelcer, E.M. Janssen, M.A. Hausner, R. Shih, J.S. Parks, P.A. Edwards, B.D. Jamieson, P. Tontonoz, LXR signaling couples sterol metabolism to proliferation in the acquired immune response, Cell 134 (2008) 97–111, https://doi.org/10.1016/j.cell.2008.04.052.

[22] J.A. Cuthbert, C.A. East, D.W. Bilheimer, P.E. Lipsky, Detection of familial hypercholesterolemia by assaying functional low-density-lipoprotein receptors on lymphocytes, N. Engl. J. Med. 314 (1986) 879–883, https://doi.org/10.1056/NEJM198604033141404.

[23] G.K. Hansson, L. Jonasson, The discovery of cellular immunity in the atherosclerotic plaque, Arterioscler. Thromb. Vasc. Biol. 29 (2009) 1714–1717, https://doi.org/10.1161/ATVBAHA.108.179713.

[24] A.J. Wilhelm, M. Zabalawi, J.M. Grayson, A.E. Weant, A.S. Major, J. Owen, M. Bharadwaj, R. Walzem, L. Chan, K. Oka, M.J. Thomas, M.G. Sorci-Thomas, Apolipoprotein A-I and its role in lymphocyte cholesterol homeostasis and autoimmunity, Arterioscler. Thromb. Vasc. Biol. 29 (2009) 843–849, https://doi.org/10.1161/ATVBAHA.108.183442.

[25] R. Chakrabarti, E.G. Engleman, Interrelationships between mevalonate metabolism and the mitogenic signaling pathway in T lymphocyte proliferation, J. Biol. Chem. 266 (1991) 12216–12222.

[26] A.J. Murphy, M. Akhtari, S. Tolani, T. Pagler, N. Bijl, C.-L. Kuo, M. Wang, M. Sanson, S. Abramowicz, C. Welch, A.E. Bochem, J.A. Kuivenhoven, L. Yvan-Charvet, A.R. Tall, ApoE regulates hematopoietic stem cell proliferation, monocytosis, and monocyte accumulation in atherosclerotic lesions in mice, J. Clin. Invest. 121 (2011) 4138–4149, https://doi.org/10.1172/JCI57559.

[27] L. Yvan-Charvet, T. Pagler, E.L. Gautier, S. Avagyan, R.L. Siry, S. Han, C.L. Welch, N. Wang, G.J. Randolph, H.W. Snoeck, A.R. Tall, ATP-binding cassette transporters and HDL suppress hematopoietic stem cell proliferation, Science 328 (2010) 1689–1693, https://doi.org/10.1126/science.1189731.

[28] T. Seijkens, M.A. Hoeksema, L. Beckers, E. Smeets, S. Meiler, J. Levels, M. Tjwa, M. P.J. de Winther, E. Lutgens, Hypercholesterolemia-induced priming of hematopoietic stem and progenitor cells aggravates atherosclerosis, FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. 28 (2014) 2202–2213, https://doi.org/10.1096/fj.13-243105.

[29] E. van Kampen, A. Jaminon, T.J.C. van Berkel, M. Van Eck, Diet-induced (epigenetic) changes in bone marrow augment atherosclerosis, J. Leukoc. Biol. 96 (2014) 833–841, https://doi.org/10.1189/jlb.1A0114-017R.

[30] J.L. Goldstein, M.S. Brown, The low-density lipoprotein pathway and its relation to atherosclerosis, Annu. Rev. Biochem. 46 (1977) 897–930, https://doi.org/10.1146/annurev.bi.46.070177.004341.