The role of abca1 in atherosclerosis: lessons from in vitro and in vivo models
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Chapter 5

Macrophage ABCA1 over-expression inhibits atherosclerotic lesion progression

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Summary
ATP-binding cassette transporter 1 (ABCA1) is a key regulator of cellular cholesterol and phospholipid transport, which is defective in familial HDL deficiency syndromes such as Tangier disease. Recently, we have shown that inactivation of macrophage ABCA1 induces atherosclerosis in LDL receptor knockout (LDLr-/-) mice. Currently, the possibly therapeutic effect of specific up-regulation of macrophage ABCA1 on atherogenesis is unknown.

Chimeras that specifically overexpress ABCA1 in macrophages were generated by transplantation of bone marrow from human ABCA1 BAC transgenic mice into LDLr-/− mice. To induce atherosclerosis, the mice were fed a Western-type diet, containing 0.25% cholesterol and 15% fat for 9 and 12 weeks, allowing analysis of effects on initial lesion development as well as advanced lesions.

Peritoneal macrophages isolated from the ABCA1 BAC → LDLr-/− chimeras exhibited a 60% (P<0.001) increase in cholesterol efflux to apoA1 as compared to controls. No significant effect of macrophage ABCA1 over-expression was observed on atherosclerotic lesion size (245±36×10^4 μm^2 in ABCA1 BAC → LDLr-/− mice versus 210±20×10^4 μm^2 in controls) after 9 weeks challenge with the Western-type diet. After 12 weeks, however, the mean atherosclerotic lesion area in ABCA1 BAC → LDLr-/− mice was only 164±15×10^4 μm^2 (P<0.001) as compared to 513±56×10^4 μm^2 in controls.

These results show that ABCA1 up-regulation does not prevent the initiation of atherosclerosis, but inhibits the progression of initial lesions to advanced atherosclerotic plaques. Therefore, the development of therapeutic targets that increase macrophage ABCA1 expression is important for the inhibition of atherosclerotic lesion progression.
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Introduction
Atherosclerotic cardiovascular disease is the major cause of morbidity and mortality in Western societies. The genesis and progression of atherosclerotic lesions involves a complicated sequence of events in which various cell types in the arterial wall, including macrophages, play an important role (1). Deposition of excessive amounts of cholesterol in macrophages leading to the transformation into foam cells is a pathological hallmark of atherosclerosis. Macrophages cannot limit their uptake of cholesterol via scavenger receptors (2). Therefore, cholesterol efflux is an important mechanism to maintain cholesterol homeostasis in macrophages and to prevent atherosclerotic lesion development. Epidemiological studies have shown a strong inverse relationship between low plasma cholesterol levels and coronary artery disease (3-5). It is currently generally accepted that high plasma levels of high-density lipoprotein (HDL) protect against the development of atherosclerosis. HDL exerts its protective effect through its role in reverse cholesterol transport, a process by which excess cholesterol from peripheral tissues is transferred via the plasma to the liver for either recycling or excretion from the body as bile (6).
A key regulator of cholesterol efflux from macrophages is ATP-binding cassette transporter 1 (ABCA1). Mutations in the human ABCA1 gene are the underlying molecular defect in familial HDL-deficiency syndromes such as Tangier disease (TD) (7-9). TD is an autosomal recessive disorder that is characterised by severe HDL-deficiency, deposition of cholesteryl esters in cells of the reticuloendothelial system, and increased susceptibility to the development of atherosclerosis (10,11).
Recently, we have shown that specific disruption of ABCA1 in macrophages results in an increased susceptibility to atherosclerotic lesion development, providing evidence that macrophage ABCA1 plays a critical role in the protection against atherosclerosis (12). Activation of this ABC transporter is thus a potentially attractive target for therapeutic interventions to prevent atherosclerosis. The expression of ABCA1 is tightly controlled by intracellular cholesterol levels (13,14). Its activity is dramatically increased upon cholesterol loading of macrophages and the subsequent transformation into foam cells. It is therefore conceivable that cholesterol efflux via ABCA1 is already maximally activated in macrophages in the atherosclerotic lesion.
To study the therapeutic potential of specific up-regulation of macrophage ABCA1 to prevent atherosclerosis, we have determined atherosclerosis susceptibility of chimeras that specifically over-express ABCA1 on macrophages, created by transplantation of bone marrow from human ABCA1 over-expressing bacterial artificial chromosome (BAC) transgenic mice to an established model of atherosclerosis. The findings from these studies revealed that specific up-regulation of macrophage ABCA1 does not affect initial lesion development, but prevents further progression of atherosclerosis.
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Methods

Mice. ABCA1 BAC 269 transgenic mice hemizygous for the human ABCA1 gene, lacking its upstream promoter and exon 1, and backcrossed to the C57BL/6 background (N6) have been generated as previously described (15,16). Non-transgenic littermates were used as controls. Homozygous LDL receptor knockout (LDLr-) mice (17) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) as mating pairs and bred at the Gorlaeus Laboratory, Leiden, The Netherlands. Mice were housed in sterilised filter-top cages and given unlimited access to food and water. They were maintained on sterilised regular chow, containing 4.3 % (w/w) fat and no cholesterol (RM3, Special Diet Services, Witham, UK), or were fed a semi-synthetic high cholesterol Western-type diet, containing 15% (w/w) fat and 0.25% (w/w) cholesterol (Diet W, Hope Farms, Woerden, The Netherlands). Drinking water was supplied with antibiotics (83 mg/L ciprofloxacin and 67 mg/L polymyxin B sulphate) and 6.5 g/L sucrose.

Bone marrow transplantation. To induce bone marrow aplasia, male LDLr- mice were exposed to a single dose of 9 Gy (0.19 Gy/min, 200 kV, 4 mA) total body irradiation, using an Andrex Smart 225 Rontgen source (XYLON International, Copenhagen, Denmark) with a 6-mm aluminium filter, one day before the transplantation. Bone marrow was isolated by flushing the femurs and tibias from male ABCA1 BAC mice or male wildtype littermates with phosphate-buffered saline. Single-cell suspensions were prepared by passing the cells through a 30-mm nylon gauze. Irradiated recipients received 0.5 x 10^6 bone marrow cells by intravenous injection into the tail vein.

Assessment of chimerism. The hematologic chimism of the LDLr- mice was determined in genomic DNA from bone marrow by PCR at 20 weeks post transplant. The forward and reverse primers 5'GGCTGGATTAGCAGTCCTCA-3' and 5'ATCCCCAACTCAAAAACCACA-3' for human ABCA1 and 5'TGGGAACCTCCTAAAAT-3' and 5'CCATGTGGTGTGTAGACA-3' for the mouse ABCA1 gene were used and resulted in 304bp and 750bp amplification products, respectively.

Macrophage cholesterol efflux studies. Thioglycollate-elicited peritoneal macrophages were harvested from mice transplanted with control and human ABCA1 BAC bone marrow at 20 weeks after transplantation and were seeded on 24-well plates at a density of 0.5 x 10^5 cells in 500 µl DMEM, supplemented with 10% (w/v) bovine calf serum (BCS), 2 mmol/l L-glutamine, 100 µg/ml streptomycin, and 100 IU/ml penicillin. After 4 hours, non-adherent cells were removed by washing. After 2 days in culture, the cells were washed and incubated with 0.5 µCi/µl H-cholesterol in DMEM/0.2% BSA for 24 hours at 37 C. Subsequently, the medium was removed and the cells were washed 3 times with DMEM/0.2% BSA. To determine cholesterol loading, cells were washed three times with washing buffer (50 mmol/l Tris, containing 0.9%
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NaCl, 1 mmol/l EDTA, 5 mmol/l CaCl₂, and 0.2% (w/v) BSA, pH 7.4), followed by two washing steps without BSA. The cells were lysed in 0.1 mol/l NaOH and the radioactivity was determined by liquid scintillation counting. Cell protein was measured according to Lowry et al. (18). Cholesterol efflux was studied by incubation of the cells with DMEM/0.2% BSA alone, or supplemented with either 10 μg/ml apolipoprotein A1 (Calbiochem) or 50 μg/ml human HDL. Radioactivity in the medium was determined by scintillation counting after 24 hours of incubation. To analyse liver X receptor (LXR) responsiveness, efflux was determined in the absence and presence of 10 μM LXRα agonist T-0901317.

**Serum lipid analyses.** After an overnight fasting-period, approximately 100 μl blood was drawn from each individual mouse by tail bleeding. The concentrations of total cholesterol, triglycerides, and phospholipids in serum were determined using enzymatic colorimetric assays (Roche Diagnostics, Mannheim, Germany). The distribution of lipids over the different lipoproteins in serum was determined by fractionation of 30 ml serum of each mouse using a Superose 6 column (3.2x30mm, Smart-system, Pharmacia, Uppsala, Sweden). Total cholesterol, triglyceride, and phospholipid contents in the effluent were determined using enzymatic colorimetric assays (Roche Diagnostics, Mannheim, Germany).

**Histological analysis of the aortic root.** To analyse the development of atherosclerosis at the aortic root, transplanted mice were sacrificed at 17 and 20 weeks post transplant and after 9 and 12 weeks of feeding the high cholesterol Western-type diet, respectively. The arterial tree was perfused in situ with phosphate-buffered saline (100 mm Hg) for 20 minutes via a cannula in the left ventricular apex. The heart plus aortic root and descending aorta were excised and stored in 3.7% neutral-buffered formalin (Formal-fixx, Shandon Scientific Ltd., England). The atherosclerotic lesion areas in oil red O-stained cryostat sections of the aortic root were quantified using the Leica image analysis system, consisting of a Leica DMRE microscope coupled to a video camera and Leica Qwin Imaging software (Leica Ltd., Cambridge, England). Mean lesion area (in μm²) was calculated from 10 oil red O-stained sections, starting at the appearance of the tricuspid valves.

**Statistical analyses.** Statistical analyses were performed utilising the unpaired student’s t-test (Instat GraphPad software, San Diego, USA).

**Results**

**Generation of LDL receptor knockout mice over-expressing macrophage ABCA1**
To assess the therapeutic potential of increasing macrophage ABCA1 to prevent atherosclerotic
lesion development, we used the technique of bone marrow transplantation to selectively up-regulate ABCA1 in hematopoietic cells. Bone marrow from previously generated human ABCA1 over-expressing BAC transgenic mice was transplanted into LDLr-/ mice, which represent an established model for the development of atherosclerosis. Successful reconstitution of recipients with hematopoietic donor cells was established at 20 weeks post transplant by PCR assisted amplification using primers specific for human and mouse ABCA1 (Figure 1A). Genomic DNA isolated from the LDLr-/ mice transplanted with bone marrow from ABCA1 BAC transgenic mice contained both the human and the mouse ABCA1 transcript whereas the control transplanted group only contained mouse ABCA1, indicating that the bone marrow transfer was successful. Furthermore, ABCA1 activity was determined by means of cholesterol efflux assays on peritoneal macrophages isolated from the transplanted mice at 20 weeks after transplantation. As previously shown for the ABCA1 BAC transgenic mice (15), macrophages isolated from LDLr-/ mice transplanted with ABCA1 BAC bone marrow exhibited a 60% (n=3, \( P<0.001 \)) increase in cholesterol efflux to apoAI compared with cells from control transplanted LDLr-/ mice (Figure 1B).

![Figure 1](image.png)

**Figure 1.** Verification of success of bone marrow transplantation. A) Verification of successful reconstitution with donor hematopoietic cells by PCR amplification of the human ABCA1 and murine ABCA1 gene at 20 weeks post transplant using genomic DNA isolated from bone marrow. B) ApoAI and HDL induced cellular cholesterol efflux from \( ^{3} \)H-cholesterol-labeled peritoneal macrophages isolated from mice transplanted with either ABCA1 BAC (n=3) or control (n=3) bone marrow at 20 weeks post transplant. Efflux to ApoAI from BAC → LDLr-/ mice showed a statistically significant difference of \( ***P<0.001 \) compared to WT → LDLr- mice.

**Effect of macrophage ABCA1 over-expression on plasma lipid levels**

During the course of the experiment, the effects of macrophage over-expression of ABCA1 on serum lipid levels were carefully monitored. On regular chow diet, the majority of the cholesterol in LDLr-/ mice is transported by LDL and HDL, phospholipids by HDL, and triglycerides by
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VLDL and LDL (Figure 2). In contrast to the ABCA1 BAC transgenic mice that displayed mildly increased HDL cholesterol levels, no significant effect on HDL cholesterol, triglyceride, or phospholipid levels was observed when ABCA1 was over-expressed solely in macrophages.

In order to induce atherosclerotic lesion development, the transplanted mice were fed a high cholesterol Western-type diet, containing 0.25% cholesterol and 15% fat, starting at 8 weeks after transplantation. Upon challenging the mice with a high cholesterol diet, serum cholesterol levels increased approximately 3-fold in both groups of mice due to an increase in VLDL and LDL cholesterol (Table I). The increase in VLDL and LDL cholesterol coincided with an increase in phospholipids. Also under these conditions, no significant effect of macrophage ABCA1 over-expression on serum lipid levels or lipid distribution over the different lipoproteins was observed (Figure 2).

**Figure 2.** Effect of macrophage ABCA1 over-expression on serum cholesterol, phospholipids, and triglyceride distribution. Blood samples were drawn after an overnight fast at 8 weeks post transplant while feeding regular chow diet (CHOW) and at 17 weeks after bone marrow transplantation after 9 weeks of feeding a high cholesterol Western-type diet (WTD). Sera from individual mice were loaded onto a Superose 6 column and fractions were collected. Fractions 3 to 7 represent VLDL; fraction 8 to 14, LDL; and fractions 15 to 19, HDL, respectively. The distribution of cholesterol, phospholipids, and triglycerides over the different lipoproteins in WT → LDLr⁻⁻ (○) and ABCA1 BAC → LDLr⁻⁻ (●) chimeras is shown. Values represent the mean±SEM of at least 12 mice. No statistically significant differences were observed.
Table 1: Effect of leukocyte ABCA1 over-expression on serum lipid levels

<table>
<thead>
<tr>
<th>Mice</th>
<th>Time (weeks)</th>
<th>Diet</th>
<th>Total Cholesterol (mg/dl)</th>
<th>HDL Cholesterol (mg/dl)</th>
<th>Phospholipids (mg/dl)</th>
<th>Triglycerides (mg/dl)</th>
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</thead>
<tbody>
<tr>
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<td>322±13</td>
<td>ND</td>
<td>524±20</td>
<td>310±34</td>
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<td></td>
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<td>140±37</td>
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<td>336±22</td>
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<tr>
<td></td>
<td>17</td>
<td>WTD</td>
<td>869±45</td>
<td>118±5</td>
<td>666±27</td>
<td>349±41</td>
</tr>
<tr>
<td>ABCA1 BAC → LDLr-/ -</td>
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<td>Chow</td>
<td>336±12</td>
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<td>893±58</td>
<td>117±4</td>
<td>721±42</td>
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</tbody>
</table>

Serum lipids were measured in LDLr-/ - mice before transplantation (baseline) and at 8 and 17 weeks after transplantation with control bone marrow or ABCA1 BAC over-expressing bone marrow. At 8 weeks after transplantation, the regular chow diet was switched to a high cholesterol Western-type diet (WTD). Data represent mean±SEM of at least 12 mice. No statistically significant differences were observed between the control transplanted group and the mice transplanted with ABCA1 BAC bone marrow.

Over-expression of macrophage ABCA1 prevents atherosclerotic lesion progression.

In order to investigate the therapeutic potential of increasing macrophage ABCA1 expression as a means of preventing atherosclerosis, we assessed whether and to what degree up-regulation of ABCA1 in macrophages affected lesion formation in the arterial wall. Atherosclerotic lesion development was analysed in the aortic root of WT → LDLr-/ - mice and in ABCA1 BAC → LDLr-/ - chimeras after 9 and 12 weeks of Western-type diet feeding. After 9 weeks on the Western diet, no significant effect of macrophage ABCA1 over-expression on the atherosclerotic lesion size was observed (245±36x10³ µm² in ABCA1 BAC → LDLr-/ - mice versus 210±20x10³ µm² in WT → LDLr-/ - mice).

![Figure 3. Macrophage ABCA1 over-expression prevents atherosclerotic lesion progression. Formation of atherosclerotic lesions was determined at 17 and 20 weeks post transplant at the aortic root of WT → LDLr-/ - and ABCA1 BAC → LDLr-/ - chimeras that were fed a high cholesterol Western-type diet for 9 and 12 weeks, respectively. The mean lesion area was calculated from oil red O-stained cross-sections of the aortic root at the level of the tricuspid valves. Values represent the mean of at least 12 mice mice. Original magnification 50x. Lesions in BAC → LDLr-/ - mice showed a statistically significant difference of ***P<0.001 when compared to WT → LDLr-/ - mice.](image-url)
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μm² in controls), indicating that macrophage ABCA1 over-expression does not prevent the initiation of atherosclerosis. From week 9 until week 12 of diet feeding, atherosclerosis in the mice transplanted with control bone marrow progressed further to advanced lesions with a mean lesion area of 513±56×10² μm². In the ABCA1 BAC → LDLr/- mice, however, the mean atherosclerotic lesion area was only 164±15×10² μm² (P<0.001), indicating that lesion progression was markedly inhibited by up-regulation of ABCA1 in macrophages. Raising macrophage ABCA1 expression is thus a promising therapeutic strategy to inhibit progression of premature atherosclerotic lesions. The identification of the nuclear receptor liver X receptor (LXR) as an activator of ABCA1 has raised the possibility of using LXR agonists to raise ABCA1 in macrophages thereby increasing cholesterol efflux (19). As shown in Figure 4, incubation of peritoneal macrophages isolated from the control transplanted mice with the synthetic LXRa agonist T-0901317 increased cholesterol efflux to ApoAI 1.8-fold (P<0.05). Although cholesterol efflux from macrophages isolated from the ABCA1 BAC → LDLr/- chimeras was already increased due to the over-expression of ABCA1, incubation with the LXR agonist further increased cholesterol efflux 2.1-fold (P<0.001).

![Figure 4. Induction of ABCA1-mediated cholesterol efflux by LXRa agonist T-0901317. ApoAI-induced cellular cholesterol efflux from ³H-cholesterol-labeled peritoneal macrophages isolated from mice transplanted with either ABCA1 BAC (n=3) or control (n=3) bone marrow was analysed for 24 hours with and without pre-incubation with 10 μM LXRa agonist T-0901317. Statistically significant difference of *P<0.05 and ***P<0.001 compared to WT → LDLr/- mice.](image)

Discussion

Insights into the roles of ABCA1 in atherogenesis have been gained from both patients affected with Tangier disease and recently developed animal models. Tangier disease, a result of mutations in ABCA1, is characterized by extremely low HDL levels, accumulation of cholesterol in macrophages, and an increased prevalence of coronary artery disease (CAD) (10-11). Interestingly, heterozygotes for ABCA1 mutations are significantly at risk for CAD (20-22). These cardioprotective
effects of ABCA1 have recently been confirmed in animal models. Overexpression of ABCA1 resulted in increased HDL cholesterol levels and a decreased susceptibility to spontaneous atherosclerotic lesion development in apolipoprotein E knockout mice (16) and in C57B1/6 mice with diet-induced atherosclerosis (23). Furthermore, using bone marrow transplantation studies, we (12) and Aello et al. (24) have shown that selective inactivation of ABCA1 in macrophages results in markedly increased atherosclerosis in different animal models.

The cardioprotective effects of ABCA1 are generally expected to be a direct result of its function in facilitating cholesterol efflux. Cholesterol efflux processes play an important role in prevention of the formation of the intimal macrophage-rich fatty streak, the first stage of atherosclerotic lesion development. Strikingly, in this study we found that over-expression of ABCA1 in macrophages did not affect initial lesion development, but rather inhibited the progression of fatty streaks into advanced lesions. In humans, fatty streaks are ubiquitous. However, not all fatty streaks evolve into advanced atherosclerotic lesions. In this study, we have demonstrated for the first time that ABCA1 is an important determinant for this progression of atherosclerosis from fatty streaks into advanced lesions.

Progression of atherosclerotic lesions is characterized by an ongoing chronic inflammatory reaction and extensive cellular necrosis and apoptosis (25). Accumulation of apoptotic markers during atherosclerosis progression indicates that in advanced lesions the removal of apoptotic cells is impaired. Several lines of evidence have suggested a role for ABCA1 in the engulfment of apoptotic cells (26). ABCA1 expression during development follows strictly the spatial and temporal pattern of programmed cell death (27). Conversely, mice deficient for ABCA1 show a defective engulfment of apoptotic corpses during development (28). Furthermore, engulfment of apoptotic thymocytes by peritoneal macrophages lacking ABCA1 is impaired (28). It is therefore conceivable that increased phagocytic activity of lesion macrophages due to ABCA1 over-expression may have led to accelerated clearance of apoptotic material thereby preventing excessive inflammatory responses and further progression of the atherosclerotic lesion.

The important role for ABCA1 in prevention of lesion progression reported in this study renders this transporter an attractive target for the development of novel therapeutic agents designed to prevent the development of advanced atherosclerotic lesions that may result in coronary or cerebral infarction. Promising candidates for raising macrophage ABCA1 expression are synthetic LXRα agonists, that induce ABCA1 expression in macrophages and can mitigate atherosclerosis in animal models (19,29).
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Contributors
M. Van Eck and R.R. Singaraja were responsible for the design of the study and wrote the paper. R.B. Hildebrand did all the bone marrow transplantation studies, including lipid analysis and atherosclerosis morphometry. E.K. James coordinated the breeding of the ABCA1 BALB/c mice. M.R. Hayden and Th.J.C. Van Berkel are the project leaders.

Conflict of interest statement
None declared

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References


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