Coexistence of Foam Cells and Hypocholesterolemia in Mice Lacking the ABC Transporters A1 and G1

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Abstract—The concept that macrophages can become foam cells as a result of a disturbed balance between the uptake of cholesterol from lipoproteins and cholesterol efflux is generally accepted. ABCA1 and ABCG1 are two cholesterol transporters that may act sequentially to remove cellular cholesterol, but currently their combined role in vivo is unknown. We report here that targeted disruption of both ABCA1 and ABCG1 in mice, despite severe plasma hypocholesterolemia, leads to massive lipid accumulation and foam cell formation of tissue macrophages. A complete ablation of cellular cholesterol efflux in vitro is observed, whereas in vivo macrophage-specific reverse cholesterol transport to the feces is markedly decreased. Despite the massive foam cell formation of tissue macrophages, no lipid accumulation was observed in the vascular wall, even in mice of 1 year old, indicating that the double knockout mice, possibly because of their hypocholesterolemia, lack the trigger to attract macrophages to the vessel wall. In conclusion, even under hypocholesterolemic conditions macrophages can be converted into foam cells, and ABCA1 and ABCG1 play an essential role in the prevention of foam cell formation. (Circ Res. 2008;102:113-120.)

Key Words: ABC transporter ■ reverse cholesterol transport ■ cholesterol efflux ■ macrophage ■ foam cell

Macrophages are implicated in the pathological deposition of cholesterol during atherogenesis as a result of the uptake of native (eg, VLDL) or modified (eg, oxidized LDL) lipoproteins1–3. Because macrophages are incapable of limiting the uptake of lipoproteins, these cells rely on cholesterol efflux mechanisms for maintaining cellular cholesterol homeostasis.4,5 In 1999, 3 groups reported that the molecular defect in Tangier Disease, an autosomal recessive disorder that is characterized by severe HDL deficiency and deposition of cholesteryl esters in cells of the reticuloendothelial system, was caused by mutations in the ABCA1 gene.6–8 Mice in which the ABCA1 gene was deleted were shown to develop a mild phenotype, we generated ABCA1/ABCG1 double knockout (dKO) mice to analyze the potential synergistic role of these transporters in mediating cellular cholesterol homeostasis.

Materials and Methods

Animals

ABCA1-deficient10 and ABCG1-deficient (Deltagen Inc, San Carlos, California) mice (both backcrossed more than 7 times on a C57BL/6J background) were mated to generate F1 heterozygotes. Heterozygote F1 animals were crossbred to obtain the ABCA1−/−/ABCG1−/− (ABCA1 KO), ABCA1−/−/ABCG1−/− (ABCG1 KO), ABCA1−/−/ABCG1−/− (dKO), and ABCA1−/−/ABCG1−/− (WT) mice. Mice were given unlimited access to food and water. Mice were maintained on sterilized regular chow, containing 4.3% (w/w) fat with no added cholesterol (RM3; Special Diet Services). Animal experiments added cholesterol (RM3; Special Diet Services). Animal experiments...
were performed at the Gorlaeus Laboratories of the Leiden/Amsterdam Center for Drug Research and Central Animal Facility of the Medical Faculty of La Pitie Hospital, Paris in accordance with the National Laws. All experimental protocols were approved by the Ethics Committee for Animal Experiments of Leiden University and the Direction Départementale des Services Vétérinaires, Paris, France, under strict compliance with European Community Regulations.

PCR and Western Blot Analysis
PCR analysis of genomic DNA was performed as described before. Immunoblotting for ABCA1 and ABCG1 protein was performed using 2 μg of protein (bone marrow–derived macrophages, see below) on a 7% (Tris Acetate, NuPage) and 10% (Bis-Tris, NuPage) gel, respectively. ABCA1 was detected using a rabbit–anti-mouse ABCA1 polyclonal antibody (Novus) as a primary antibody. ABCG1 was detected using a rabbit–anti-mouse ABCG1 polyclonal antibody (Novus) as a primary antibody. As a secondary antibody a goat–anti-rabbit IgG-HRP (Jackson) was used.

Lipid Analyses
Blood was collected by retro-orbital puncture under anesthesia. The concentrations of total cholesterol in plasma were determined as described before. Plasma HDL cholesterol levels were determined by fractionation of 50 μL of plasma from each mouse using a Superose 6 column (3.2×300 mm, Smart-System; Pharmacia).

Peritoneal Leukocyte Analysis
On sacrifice the peritoneal cavity of the mice was lavaged with 10 mL cold PBS to collect peritoneal leukocytes for quantification of macrophage foam cells using an automated Sysmex XT-2000iV Veterinary Hematology analyzer (Sysmex Corporation). Corresponding samples were cytopspun for manual confirmation and stained with Oil red O for detection of lipid accumulation, and counterstained with hematoxylin.

Histological and Tissue Lipid Analysis
Histological analyses were performed as described before on seven micrometer cryosections of lung, liver, thymus, and Peyer patches. In addition, a fragment of intestine containing a Peyer Patch was cut longitudinally on the opposite side of the Peyer Patch and stained with Oil red O. Hepatic lipids were extracted as described earlier. Hepatic lipids were extracted as described earlier.21

Atherosclerotic lesion development was quantified in the aortic root of dKO mice of 1 year old as described previously.20

Cellular Cholesterol Efflux Assays
For each mouse, bone marrow cells were isolated from both the femurs and tibias by lavage with phosphate-buffered saline (PBS). Cells were 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 5 days. Bone marrow–derived macrophages (BMDM) were then cholesterol-loaded for 48 hours with acetylated LDL (50 μg/mL) in DMEM containing BSA (0.1%; wt/vol). The loading medium was removed and the cells washed twice in PBS, then incubated overnight (16 hours) with DMEM containing BSA (0.1%; wt/vol), supplemented with 22-hydroxycholesterol (10 μmol/L) and 9-cis retinoic acid (1 μmol/L). To measure cholesterol efflux, cells were incubated in DMEM/0.1% BSA alone or with lipid-free apoAI (10 μg protein/mL) or HDL2 (10 μg protein/mL) for 24 hours. At 24 hours the media were removed and the cells lysed in 0.1N NaOH. Cell and media samples were also extracted and analyzed for free and esterified cholesterol mass by HPLC, as previously described.22 Cell proteins were measured using the BCA assay (Pierce). For mass analysis, HDL samples were separately analyzed to allow correction for HDL cholesterol present in relevant media samples. Cholesterol efflux is expressed as the percentage of total cell cholesterol present in the medium. Basal efflux to media (in the absence of added acceptors) was subtracted from the data shown.

In Vivo Macrophage-Specific Reverse Cholesterol Transport Assay
BMDM were prepared as described above and loaded with 50 μCi/mL [3H]-cholesterol-labeled acetylated LDL (5 μCi/mL) in fresh differentiation media containing 4 μg/mL 22-hydroxy-cholesterol and 1 μmol/L 9-cis retinoic acid for 48 hours. [3H]-cholesterol labeled-BMDM were washed twice in PBS and harvested by treatment with acetate (PAA) for 15 minutes at 37°C. Cells were spun down at 4°C and resuspended in cold PBS. Radioactivity incorporated in BMDM was determined by double extraction in hexane-isopropanol (3:2), evaporation of the solvent and liquid scintillation counting (Beckman). Male C57BL/6J mice were fed a standard chow diet and housed in separate cages. Mice were injected intraperitoneally with 5×106 [3H]-cholesterol-labeled BMDM in 0.5
mL PBS. Blood was collected at 24 hours after injection by retro-orbital puncture under isoflurane anesthesia and radioactivity in plasma was measured by liquid scintillation counting (Beckman). Mice were euthanized by cervical dislocation, perfused transcardially with PBS and livers were removed and weighed. Radioactivity in homogenized liver samples was counted directly in a scintillation vial (Beckman). Feces were collected, dried at 50°C, weighed and rehydrated at a similar concentration overnight. Fecal samples were then homogenized and radioactivity determined by liquid scintillation counting (Beckman). The amount of [3H]-tracer in plasma, liver, and feces was expressed as a percent of the injected dose.

Statistical Analysis
Statistical analysis between the 4 groups was performed using ANOVA and the Student-Newman-Keuls post-test (GraphPad InStat and Prism software). Statistical analysis on reproduction was performed using the X^2-test (GraphPad InStat and Prism software).

Results
To analyze the potential synergistic role of the transporters ABCA1 and ABCG1 in mediating cellular cholesterol homeostasis we generated ABCA1/ABCG1 double knockout (dKO) mice. In Figure 1 the verification of the absence of ABCA1 and ABCG1 in the dKO mice is shown at both the DNA and protein level by performing PCR and Western blot analysis, respectively.

Figure 2. The hypocholesterolemia of ABCA1/ABCG1 dKO mice is associated with abnormal morphology of liver, spleen, and intestinal Peyer patches. A, Plasma total cholesterol and HDL-cholesterol levels in WT, ABCA1 KO, ABCG1 KO, and ABCA1/ABCG1 dKO animals show that ABCA1/ABCG1 dKO mice are severely hypocholesterolemic and hypoalphalipoproteinemic. B, Compared with wild-type and single knockout mice hepatic triglyceride and phospholipid levels (expressed as µg/µg liver protein) did not differ between ABCA1/ABCG1 dKO mice, whereas free and esterified cholesterol concentrations were increased. The liver (C) and spleen (D) of the dKO mice contained numerous pale white foci; (E) the Peyer patches lay like “white marbles” on top of intestinal tissue, (F) which stained positive for Oil red O, indicating neutral lipid accumulation. Data presented as mean±SEM, n=4 to 14/genotype, ***P<0.001.
reach the age of >1 year. No significant differences in body weight between WT, ABCA1 KO, ABCG1 KO, and dKO mice were observed at the age of 6 months (22.1±1.6 g, 23.3±0.3 g, 22.6±0.8 g, 20.2±0.9 g, respectively; n=4). In contrast to ABCG1 knockout (KO) mice whose plasma cholesterol and HDL cholesterol concentrations are similar to those of wild-type (WT) mice,15 ABCA1 KO,10 and dKO mice are severely hypocholesterolemic (Figure 2A). Indeed, when fed a regular chow diet, plasma total cholesterol and HDL-cholesterol concentrations were decreased 80% in dKO mice (Figure 2A). Despite low serum cholesterol levels, ABCA1/ABCG1 dKO mice showed profound changes in tissues rich in macrophages, including the liver, spleen, lung, thymus, lymph nodes, and the Peyer patches. Furthermore, severe hepatosplenomegaly and enlargement of lymph nodes and Peyer patches was observed in dKO mice, whereas no such phenotype was evident in the ABCA1 and ABCG1 single knockout or wild-type animals. The liver (Figure 2C) and spleen (Figure 2D) of the dKO mice contained many pale white foci, whereas the Peyer patches presented as “white marbles” on top of the intestine (Figure 2E and 2F), such morphology is consistent with lipid accumulation. Consequently, cryostat sections of liver, spleen, and Peyer patches showed massive neutral lipid accumulation, as indicated by Oil red O staining (Figure 3). Excessive neutral lipid accumulation was not limited to the liver, spleen, and Peyer patches however, as Oil red O staining was also consistently greater in thymus (Figure 3) and lymph nodes (not shown) of dKO mice as compared with wild-type and single knockout animals. Within the different tissues of the dKO mice fed a chow diet, Oil red O staining was mainly observed in macrophage-rich areas like the red pulp of the spleen and thymus, as evidenced by colocalization of Oil red O with MOMA-2 macrophage staining (data not shown). Consistent with this finding, Oil red O staining was absent from kidney, a tissue poor in macrophages (data not shown). Furthermore, virtually no Oil red O staining was observed in the liver, spleen, Peyer patches, and thymus of WT, ABCA1 KO, and ABCG1 KO mice on a chow diet. The only organ showing lipid accumulation in single ABCA1 KO and single ABCG1 KO mice were the lungs, in agreement with earlier findings.10,15 However, in the ABCA1/ABCG1 dKO mice, the lipid accumulation was scattered more widely throughout the lung. The foci of neutral lipid accumulation in the liver of dKO mice were concluded to comprise free cholesterol and esterified cholesterol, and not triglycerides or phospholipids, on the basis of selective accumulation of free cholesterol and esterified cholesterol in liver extracts (Figure 2B).

We subsequently examined the effect of combined deletion of ABCA1 and ABCG1 on foam cell formation within the peritoneal cavity by isolating resident peritoneal leukocytes. The collected cells were analyzed using an automated hema-

**Figure 3.** ABCA1 and ABCG1 are crucial for maintaining cholesterol homeostasis in tissues rich in macrophages. Cryostat sections of individual tissues were prepared and stained with Oil red O for lipid visualization. For the dKO mice 3 different magnifications (see lower left of each image) from the same tissue areas are displayed. Massive neutral lipid accumulation was observed in the liver, spleen, lung, Peyer patches, and thymus of dKO mice, whereas no such phenotype was evident in the single knockout and wild-type animals, with the exception of the lung of ABCA1 and ABCG1 KO mice. No Oil red O staining was observed in the kidney, a tissue poor in macrophages (data not shown).
ology analyzer with 5-differential leukocyte population counting. The resulting scattergrams are shown in Figure 4A. Interestingly, the isolated peritoneal leukocytes from the dKO animals reveal a group of cells that is shifted to the upper-right of the plot when compared with WT, ABCA1 KO, and ABCG1 KO animals, thereby indicating that this group of cells was larger and contained more abundant granules. To determine whether the increased granularity resulted from enhanced foam cell formation, collected cells were cytospun and stained for lipids with Oil red O (See Figure 4B and 4C for representative photomicrographs). In agreement with the observed shift of the macrophage population in dKO animals, lipid-laden peritoneal cells were more numerous as compared with the corresponding populations in WT, ABCA1 KO, and ABCG1 KO mice (Figure 4D).

ABCA1 is involved in the efflux of cholesterol from peripheral tissue macrophages to lipid-free apolipoproteins, and in particular to apoAI,6,7,23 whereas ABCG1 facilitates cellular cholesterol efflux from macrophages to lipided particles such as mature HDL, but not to lipid-free apolipoproteins.13–15 To directly test the consequence of combined ABCA1 and ABCG1 deficiency on cholesterol efflux, mass efflux experiments were performed. Bone marrow–derived macrophages from WT, ABCA1 KO, ABCG1 KO, and dKO mice were cholesterol-loaded with acetylated LDL, and cholesterol mass efflux to HDL or apoAI was determined. Consistent with a role for ABCA1 in mediating the efflux of cholesterol to lipid-poor apolipoproteins,6,7,23 and for ABCG1 in mediating the efflux of cholesterol to HDL,13–15 mass efflux of cholesterol from macrophages of ABCA1 KO mice to apoAI was almost completely inhibited, whereas mass efflux of cholesterol from macrophages of ABCG1 KO mice to HDL was significantly reduced (−32%; Figure 5A and 5B). Whereas single ABCG1 deficiency did not affect mass cholesterol efflux to apoAI, ABCA1 deficiency led to reduced cholesterol mass efflux to HDL. Most importantly, cholesterol mass efflux to both apoAI and HDL was virtually absent in macrophages isolated from the dKO mice.

The relative importance of ABCA1 and ABCG1 in promoting in vivo lipid efflux from macrophages, and thus their effect on attenuating foam cell formation, was further tested by using a macrophage-specific reverse cholesterol transport (RCT) assay.24 By transfer of [3H] cholesterol–labeled bone marrow–derived macrophages to the peritoneal cavity of control C57Bl/6J mice with normal serum lipoprotein levels, the release of [3H]-cholesterol from macrophages obtained from WT, ABCA1 KO, ABCG1 KO, and dKO mice to the plasma and their transport to the liver and ultimately the feces can be quantified. At 24 hours after injection of the [3H]-cholesterol loaded macrophages into the peritoneal cavity, plasma radioactivity derived from the dKO macrophages was significantly lower, while the amount of [3H]-tracer found in

![Figure 4. Accumulation of heavily lipid-laden macrophage foam cells in the peritoneal cavity of ABCA1/ABCG1 dKO mice. Peritoneal leukocytes were analyzed using an automated Sysmex XT-2000iV Veterinary Hematology analyzer. A, Scattergrams of peritoneal leukocytes from WT, ABCA1 KO, ABCG1 KO, and dKO mice. B and C, Photomicrographs of cytospins of peritoneal cells of the corresponding animals after Oil red O staining. Original magnification 5×10 and 40×10, respectively. D, Quantification of macrophage foam cells as percentage of the total number of isolated cells. Note the massive lipid accumulation in peritoneal leukocytes from dKO mice. Data presented as mean±SEM, n=3 to 7/genotype, ***P<0.001.](http://circres.ahajournals.org/ Downloaded from)
the liver, and especially the amount of tracer excreted into the feces, was significantly less as compared with animals that received macrophages from WT, ABCA1 KO, and ABCG1 KO mice (Figure 5C, 5D, and 5E).

Finally, we analyzed the effect of double ABCA1 and ABCG1 deficiency on lipid deposition in the arterial wall. Interestingly, despite massive lipid accumulation in macrophages of different organs, dKO mice did not show any atherosclerotic lesion development (Figure 6). Thus, macrophage lipid-loading is only seen in macrophage-containing organs of double knockouts, while the dKO mice lack the high plasma cholesterol levels needed to trigger arterial wall accumulation of macrophages.

**Discussion**

In the current study we show that on a regular chow diet the combined deletion of ABCA1 and ABCG1 in mice does lead to massive lipid accumulation in peritoneal macrophages as well as macrophage-rich tissues like the liver and spleen, leading to severe hepatosplenomegaly. Furthermore, lymph nodes and Peyer patches were increased in size and display massive lipid-loading in macrophage-rich areas. No such phenotype was evident in the ABCA1 and ABCG1 single knockout or wild-type animals. Single ABCG1 KO mice only showed Oil red O staining in multiple tissues and elevated hepatic concentrations of cholesterol, triglycerides, and phospholipids on challenge with a high-fat/high-cholesterol diet for 9 weeks leading to serum cholesterol values of 140 to 170 mg/dL. This phenotype is however less severe as compared with that of ABCG1/ABCA1 dKO mice on a chow diet, whose serum cholesterol levels are only 10 mg/dL. The massive formation of tissue macrophage foam cells, despite severe hypocholesterolemia, clearly illustrates the essential combined role of these transporters in maintaining macrophage cellular cholesterol homeostasis.

Interestingly, it also suggests that other efflux mechanisms cannot compensate for the absence of these 2 transporters. Three genes are suggested to play a role in cholesterol efflux from macrophages: ABCA1, ABCG1, and SR-B1. ABCA1 stimulates cholesterol and phospholipid efflux from macro-
LXR-stimulated C57BL/6J mice led to a 25% decrease in deficiency of ABCA1 and ABCG1 in J774 cells transferred to macrophage reverse cholesterol transport, combined partial phage SR-BI was shown not to be involved in promoting specific in vivo reverse cholesterol transport. Whereas macrophages to lipid-poor apoAI, but not to mature HDL.26 ABCG1 has been implicated in the efflux of cholesterol to mature HDL.13–15 SR-BI requires a phospholipid-containing acceptor, like mature HDL to induce a concentration gradient dependent efflux of free cholesterol.27,28 In vitro studies showed that the transfer of lipids to apoAI mediated by ABCA1 activity is sufficient to generate an efficient acceptor for ABCG1-mediated cholesterol efflux, which implies that ABCA1 cooperatively works with ABCG1 in cholesterol transport.16,17 The observed dramatic enhancement of macrophage foam cell formation as a result of combined deletion of ABCA1 and ABCG1 as compared with single deletion of ABCA1 and ABCG1 in this study would indeed favor this proposed model, while SR-BI could not prevent the accumulation of lipids. However, SR-BI might efflux from a specific compartment or functionally distinct cellular pools of cholesterol other than ABCG1.29

During the preparation of our manuscript, an article of Wang et al.30 appeared, describing the role of ABCA1, ABCG1, and SR-BI in in vitro efflux and in macrophage-specific in vivo reverse cholesterol transport. Whereas macrophage SR-BI was shown not to be involved in promoting macropage reverse cholesterol transport, combined partial deficiency of ABCA1 and ABCG1 in J774 cells transferred to LXR-stimulated C57BL/6d mice led to a 25% decrease in reverse cholesterol transport from macrophages to the feces.30 We used WT, ABCA1, ABCG1, and dKO mice bone marrow–derived macrophages with the same genetic background as the recipient mice and observed that the combined complete deficiency of ABCA1 and ABCG1 led to a striking 71% decrease in fecal excretion of [3H]-tracer. Also the plasma and liver [3H]-tracer in mice injected with dKO macrophages were significantly decreased compared with mice injected with WT, ABCA1, or ABCG1 KO macrophages, indicating that the reverse cholesterol transport pathway in the complete absence of ABCA1 and ABCG1 was highly impaired.

In this study, we furthermore choose to determine the effect of combined deletion of ABCA1 and ABCG1 on the net efflux of cholesterol by performing mass efflux experiments. It is known from previous studies that absence of ABCA1 results in a decreased efflux of cholesterol from macrophages toward apoAI.26 Accordingly, compared with WT and ABCG1 KO macrophages, we found a 72% decrease in net cholesterol efflux from ABCA1 KO macrophages, whereas in dKO cells the efflux was decreased even slightly more (84%). In agreement with the suggested role for ABCG1 in the efflux of cholesterol to mature HDL13–15 we also found a significant decrease in the net cholesterol efflux from ABCG1 KO macrophages to HDL. In the absence of both ABCA1 and ABCG1 expression, we did observe a complete abolishment of mass cholesterol efflux from dKO macrophages, indicating the essential role of the combined presence of ABCA1 and ABCG1 for cholesterol release from macrophages to HDL. Thus we conclude that the highly impaired efflux capacity of macrophages from dKO mice is likely to be responsible for the massive accumulation of lipids in tissue macrophages observed in dKO mice. Despite the massive foam cell formation of tissue macrophages lacking ABCA1 and ABCG1, no lipid accumulation was observed in the vascular wall. Thus, probably because of the absence of high plasma cholesterol levels no stimulus is available to attract macrophages to the arterial wall thereby preventing atherosclerotic lesion development.

Administration of the LXR agonist GW3965, which induces both ABCA1 and ABCG1 expression, has been shown to increase the rate of RCT from macrophages to feces in vivo.24 The present studies reveal that the combined action of ABCA1 and ABCG1, both key LXR target genes, is critical to maintaining lipid homeostasis in the macrophage. Despite the fact that no atherosclerotic lesion development was observed in dKO mice, targeted upregulation of ABCA1 and ABCG1 expression, for instance by nuclear receptor agonists, may constitute an effective pharmacological approach to the prevention of vascular lipid accumulation.

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Disclosures
None.

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