Stimulation of Cholesterol Efflux by LXR Agonists in Cholesterol-Loaded Human Macrophages Is ABCA1-Dependent but ABCG1-Independent

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Objective—Maintenance of cholesterol homeostasis in human macrophages is essential to prevent foam cell formation. We evaluated the relative contribution of the ABCA1 and ABCG1 transporters to cholesterol efflux from human macrophages, and of the capacity of LXR agonists to reduce foam cell formation by stimulating export of cellular cholesterol. Methods and Results—ABCG1 mRNA levels were strongly increased in acLDL-loaded THP-1 macrophages and in HMDM on stimulation with LXR agonists. However, silencing of ABCG1 expression using ABCG1-specific siRNA indicated that ABCG1 was not essential for cholesterol efflux to HDL in cholesterol-loaded human macrophages stimulated with LXR agonists. Indeed, ABCA1 was solely responsible for the stimulation of cholesterol efflux to HDL on LXR activation, as this effect was abolished in HMDM from Tangier patients. Furthermore, depletion of cellular ATP indicated that the LXR-induced export of cholesterol was an ATP-dependent transport mechanism in human macrophages. Finally, use of an anti–Cla-1 blocking antibody identified the Cla-1 receptor as a key component in cholesterol efflux to HDL from cholesterol-loaded human macrophages. Conclusion—Our data indicate that stimulation of cholesterol efflux to HDL by LXR agonists in human foam cells involves an ATP-dependent transport mechanism mediated by ABCA1 that it appears to be independent of ABCG1 expression. (Arterioscler Thromb Vasc Biol, 2009;29:00-00.)

Key Words: ●●●

In atherosclerotic lesions, the accumulation of cellular cholesterol within macrophage “foam cells” drives lipid deposition and is a major contributor to lesion growth. It is understood that macrophages become foam cells as the result of a loss of the normal balance between cholesterol uptake (from lipoproteins) and cholesterol export. For this reason, the mechanisms by which macrophages export cellular cholesterol have been intensively investigated in recent years. It is now generally accepted that HDL and its apolipoproteins are the major initial acceptors of excess cellular cholesterol. Members of the ABC transporter family have been identified as key, and potentially complementary, cellular participants in export of cholesterol to these acceptors. ABCA1 mediates cholesterol efflux most efficiently to lipoprotein-Apolipoprotein A1 (apoAI), whereas ABCG1 promotes cholesterol export to lipoparticle such as HDL. In addition, the SR-BI receptor pathway can also promote cholesterol export to HDL particles. The contributions of ABCA1 and ABCG1 to the maintenance of macrophage cholesterol homeostasis have been most convincingly demonstrated through the effects of targeted deletion of these transporters in mice.1 In particular, the profound effects of combined deletion of both ABCA1 and ABCG1 in mouse macrophages on their accumulation of cholesterol in vivo and on their ability to export cholesterol to either apoAI or to HDL in vitro, suggests that the combined activity of both of these transporters is essential for macrophage cholesterol homeostasis.2,3 However, there is substantial evidence that regulation of the expression of some key genes involved in cholesterol homeostasis, as well as the signaling pathways controlling foam cell formation, differ in a species-specific manner.

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between mouse and human. Such distinctions highlight the need to further explore mechanisms implicated in cholesterol homeostasis specifically in human macrophages. The contribution of human ABCA1 in cholesterol homeostasis and in HDL metabolism is already established through the identification of ABCA1 mutations in Tangier Disease. In contrast, no functional mutations of ABCG1 or Cla-1 (the human homologue of SR-BI) have yet been identified in human subjects. Polymorphisms in the Cla-1 gene are associated with plasma HDL-C and LDL-C levels and with incidence of coronary artery disease, but do not indicate a specific role for Cla-1 in macrophage lipid homeostasis. Similarly, a correlation between ABCG1 expression and cholesterol efflux was reported in macrophages from patients with type 2 diabetes mellitus, although a direct causal relationship remains to be demonstrated.

The liver X receptors, LXRs, are nuclear receptors that act as cholesterol sensors in controlling transcription of genes involved in cholesterol homeostasis and lipid metabolism. LXR agonists have been demonstrated to delay atherosclerosis in mice by stimulating reverse cholesterol transport from macrophages. Additional studies have revealed that expression of LXR in macrophages is a prerequisite for the atheroprotective effect of LXRs. Because ABCG1 and ABCA1 gene expression is stimulated by LXR agonists in human macrophages, upregulation of macrophage ABCG1 and ABCA1 by LXR agonists may constitute an effective pharmacological approach to attenuate foam cell formation, and thereby to prevent arterial lipid accumulation and lesion progression in dyslipidemic patients.

Our present objective was to evaluate the relative contributions of ABCG1, ABCA1, and Cla-1 to cholesterol efflux from human macrophages and to determine their respective roles in the stimulatory effects of LXR agonists on cholesterol efflux from human macrophages. Surprisingly, we demonstrate that efflux of cholesterol from human foam cell macrophages to HDL is independent of ABCG1 expression but specifically requires expression of ABCA1. Cla-1 also contributes to cholesterol efflux to HDL from cholesterol-loaded human macrophages, independently of LXR stimulation. These findings indicate that the relative contributions of the ABCA1, ABCG1, and Cla-1 pathways to cholesterol efflux from human macrophages differ significantly from those in mouse. Future investigations of the pharmacological modulation of cholesterol homeostasis in monocyte-macrophages must therefore be focused on appropriately humanized mouse models of atherosclerosis.

### Materials and Methods

Human THP-1 monocytic cells were obtained from the American Type Culture Collection (ATCC) and maintained in RPMI 1640 medium, supplemented with 10% heat-inactivated human serum, 2 mmol/L glutamine, and 100 U/mL penicillin/streptomycin at 37°C in 5% CO2. THP-1 were differentiated into macrophage-like cells with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) for 3 days. Mono- cytes were isolated from the blood of individual healthy normolipemic donors (Établissement Français du Sang, EFS), or from a Tangier Disease (TD) patient by Ficoll gradients (Ficoll-Paque PLUS, GE Healthcare) and subsequently differentiated into human macrophages (HMDM) by adhesion on plastic Primaria plates (Falcon) over a period of 10 days of culture in RPMI 1640 medium, supplemented with 10% heat-inactivated human serum, 2 mmol/L glutamine, 100 µg/mL penicillin/streptomycin, and 20 ng/mL human macrophage colony-stimulating factor (h-CSF).

Details of radioisotopic and mass cholesterol efflux assays, Western blot analysis, RNA interference (RNAi)-mediated ABCG1 silencing using small interference (si)RNA, stable knockdown of ABCG1 in THP-1 macrophages, RNA extraction, reverse-transcription and quantitative-PCR, and statistical analyses are available in the supplemental materials (please see http://atvb.ahajournals.org).

### Results

#### ABCG1 Is Highly Expressed in Cholesterol-Loaded Human Monocyte-Derived Macrophages on Stimulation by LXR Agonists

The capacity of natural LXR/RXR agonists (22OH-C/9cRA), synthetic LXR agonist TO901317, and 8BrcAMP to stimulate the expression of ABCA1, ABCG1, and Cla-1 genes in human macrophages (THP-1 and HMDM) are described in detail in the supplemental materials.

We determined the impact of such LXR agonists on the expression of ABCG1 relative to that of ABCA1 and to Cla-1 in human macrophages (THP-1 and HMDM), and thus on their potential contribution to cholesterol homeostasis in macrophages and foam cells. In the basal state, levels of both ABCG1 and ABCA1 mRNA were substantially lower than those of Cla-1 in both THP-1 macrophages and HMDM (Figure 1), thereby indicating that the Cla-1 receptor was consistently expressed at high levels in human macrophages (THP-1 and HMDM). However, cholesterol loading of human macrophages with acLDL led to a marked increase in mRNA levels of ABCA1 (THP-1: 6.4-fold and HMDM: 4-fold, \( P < 0.005 \)) and ABCG1 (THP-1: 4.6-fold, \( P < 0.005 \) and HMDM: 8.6-fold, \( P < 0.0005 \)), whereas those of Cla-1 were unchanged.

Stimulation of acLDL-loaded human macrophages with the natural LXR/RXR agonist (22OH-C/9cRA), or the synthetic LXR agonist TO901317, further enhanced the expression of both the ABCA1 and ABCG1 genes, with the highest amounts of mRNA observed for the ABCG1 gene. Interestingly, the addition of either 22OH-C/9cRA or TO901317 induced a 1.7-fold and 1.6-fold increase in Cla-1 mRNA levels in acLDL-loaded HMDM, respectively, but was without effect on acLDL-loaded THP-1 macrophages. Taken together, these results indicate that Cla-1 mRNA levels were more abundant than those of ABCA1 and ABCG1 in human macrophages in the basal noncholesterol loaded state. However, ABCG1 mRNA levels predominated in acLDL-loaded macrophages stimulated with LXR agonists, thereby suggesting a role of ABCG1 in maintaining cholesterol homeostasis in cholesterol-laden human macrophages.

#### Elevated ABCG1 Gene Expression Is Associated With Elevated Cellular Cholesterol Efflux to HDL in Human Macrophages

Because ABCG1 has been reported to mediate cholesterol efflux to HDL in mouse macrophages, one would expect that the marked elevation in ABCG1 expression in human macrophages on stimulation with LXR agonists would lead to a concomitant stimulation of cholesterol efflux to HDL.
As shown in Figure 2A, the stimulation of human macrophages by LXR agonists was accompanied by an increase of cellular [3H]cholesterol efflux to HDL (22OH-C/9cRA: 1.7-fold, \(P < 0.0005\) and 1.8-fold, \(P < 0.005\); TO901317: 1.4-fold, \(P < 0.0005\)), in all likelihood as the consequence of the phosphorylation of ABCA1 by the cAMP/PKA pathway, which may directly promote cellular cholesterol efflux.12

Consistent with levels of ABCA1 expression observed in human macrophages (Figure 1), [3H]cholesterol efflux to apoAI was robustly induced on stimulation with 22OH-C/9cRA (2.4-fold and 2-fold, \(P < 0.0005\)) or TO901317 (2.6-fold and 2.5-fold, \(P < 0.0005\)) in THP-1 macrophages and HMDM, respectively. Although ABCA1 mRNA levels were either unchanged or poorly elevated in the presence of 8Br-cAMP, [3H]cholesterol efflux to apoAI in HMDM was enhanced by 8Br-cAMP (1.4-fold, \(P < 0.0005\)), suggesting that signaling pathways requiring cAMP do not contribute to cholesterol efflux to HDL.

In agreement with results obtained from the analysis of cholesterol efflux to either lipid-free apoAI or to HDL particles, the capacity of human macrophages to promote cellular cholesterol efflux to whole human plasma containing cholesterol acceptors (HDL, apoAI) at physiological levels was also increased on stimulation with 22OH-C/9cRA (+50% and +70%, \(P < 0.0005\)) or TO901317 (+30%, \(P < 0.0005\)) in both acLDL-loaded THP-1 macrophages and HMDM, respectively (Figure 2C).

### Cellular Cholesterol Efflux to HDL in Cholesterol-Loaded Human Macrophages Is ABCG1-Independent

To determine whether the induction of cholesterol efflux to HDL by LXR agonists results from stimulation of ABCG1 expression, and equally to determine the potential contribution of ABCG1 to mechanisms of cholesterol efflux in human macrophages, we silenced ABCG1 gene expression using siRNA specific for the human ABCG1 gene. As compared to control siRNA, siRNA targeting human ABCG1 led to a complete abolition of ABCG1 expression in nonstimulated HMDM as measured by quantification of protein levels (Figure 3A). In addition, silencing of ABCG1 expression (ABCG1 Knockdown [kDa]) completely abolished the strong stimulation of ABCG1 expression by LXR/RXR agonists (22OH-C/9cRA) and led to a strong inhibition of ABCG1 mRNA and protein expression (Figure 3B).

![Figure 1. Abundance of ABCG1 mRNA levels in cholesterol-loaded human macrophages stimulated with LXR agonists. Relative quantification of ABCA1, ABCG1, and Cla-1 mRNA levels in THP-1 macrophages (A) and HMDM (B) by real-time quantitative PCR. Values are means ± SEM. C, Summary of data presented in A and B.](http://arh.ahajournals.org/)

![Figure 2. Marked stimulation of cholesterol efflux from human macrophages by LXR agonists. Cholesterol efflux to HDL (A), lipid-free apoAI (B), or normolipidemic plasma (C) from cholesterol-loaded THP-1 macrophages and HMDM. Values are means ± SEM and are expressed as relative to cells incubated without agonists. *P < 0.05 and **P < 0.005 compared with untreated cells.](http://arh.ahajournals.org/)
terol efflux assays, from acLDL-loaded HMDM enriched in CE (CE: 48.2 ± 1.9% and FC: 51.8 ± 1.9% of total mass cholesterol as measured by HPLC). In contrast, a marked reduction of cholesterol efflux to HDL (−40%, P < 0.05) was observed from LXR-induced, cholesterol-loaded, bone marrow–derived macrophages (BMDM) from ABCG1 KO mice relative to WT BMDM (Figure 3E).

The absence of such an effect in human macrophages was also observed in THP-1 macrophages transiently transfected with specific siRNA targeting human ABCG1, in which ABCG1 protein expression was reduced by 90% (supplemental Figure II). Finally, to further confirm that the silencing of ABCG1 expression in human macrophages was without effect on cholesterol efflux to HDL, several THP-1 clones stably transfected with a shRNA targeting human ABCG1 (in which ABCG1 protein expression is stably knocked down (ABCG1 SKD) were compared. We chose 3 clones, in which ABCG1 protein expression was either completely undetectable (clones 1 and 3) or only barely detectable after LXR induction (clone 2; Figure 3F). ABCA1 expression (Figure 3F) and cholesterol efflux to ApoAI (Figure 3G) was markedly and equally stimulated by LXR ligands in all 3 ABCG1-silenced THP-1 clones and in Ctrl THP-1 cells. However, and in keeping with our results in silencing ABCG1 in HMDM, cholesterol efflux to HDL was not decreased, even in the complete absence of detectable ABCG1 protein in ABCG1 SKD THP-1 macrophages (Figure 3H).

As the absence of ABCG1 may lead to an increase of ABCA1 protein expression in ABCG1 KO mice, we explored the possibility that the knockdown of ABCG1 activity could be compensated by upregulation of ABCA1 expression in our cell systems. However, analysis of ABCA1 protein levels in ABCG1 SKD THP-1 macrophages (Figure 3F) and in ABCG1 kDa HMDM (supplemental Figure IIIA) indicated that ABCA1 protein expression was not significantly or consistently upregulated as a result of ABCG1 knockdown.

Taken together, our data indicate that, in contrast to mouse macrophages, the deletion of the ABCG1 transporter does not detectably reduce cholesterol efflux to HDL from cholesterol-loaded human macrophages.

**Figure 3.** Stimulation of cholesterol efflux to HDL by LXR agonists does not require the ABCG1 transporter in cholesterol-loaded human macrophages. A and F, Total ABCG1 protein was assessed by Western blot analysis. B, The efficiency of the ABCG1 knockdown in HMDM was assessed by quantification of mRNA and protein levels. ABCG1 mRNA was normalized to housekeeping genes (β-aminolevulinate synthase and human β-tubulin) and ABCG1 protein to matching -actin levels. Mass (D) and radioisotopic cholesterol efflux to HDL (C, E, and H) or lipid-free ApoAI (G) was assayed in cholesterol-loaded macrophages either in HMDM transiently transfected with control siRNA (Ctrl); or siRNA targeting ABCG1 (ABCG1 kDa; C and D); or THP-1 stably transfected with shRNA targeting human ABCG1 (ABCG1 SKD; G and H); or alternatively in TO901317-stimulated WT and ABCG1 KO BMDM (E). Values are means ± SD. *P < 0.05 compared to control cells.

**Stimulation of Cholesterol Efflux to HDL by LXR Agonists Requires the ABCA1 Transporter in Cholesterol-Loaded Human Macrophages**

Because the LXR-mediated stimulation of cholesterol efflux to HDL in cholesterol-loaded human macrophages is independent of ABCG1 expression, we next tested the possibility that the latter results from the induction of ABCA1 expression by LXR agonists. To address this possibility, cholesterol efflux to HDL was evaluated in HMDM from a Tangier Disease (TD) patient displaying a functional homozygous mutation in the ABCA1 gene. As expected, cholesterol efflux to lipid-free apoAI was almost completely abolished in cholesterol-loaded TD HMDM as compared to control HMDM (Figure 4A). In addition, cholesterol efflux to HDL was lower (−25%, Figure 4B) in TD HMDM than in control HMDM as previously observed in fibroblasts from TD patients.13

More strikingly, incubation with LXR/RRX agonists (22OH-C/9cRA) led to a significant stimulation of cholesterol efflux to HDL in control HMDM (P < 0.05), whereas no effect was observed in TD HMDM (Figure 4B). These findings clearly indicate that the induction of free cholesterol export to HDL by LXR agonists involves an ABCA1-dependent pathway in cholesterol-loaded human macrophages.

Additional experiments presented in detail in supplemental materials confirmed that the HDL preparation used in our studies did not contain species (such as lipid-free apoAI) that can directly promote cholesterol efflux via ABCA1. How-
ever, we cannot exclude the possibility that such species are generated from HDL when it is incubated with human macrophages.

Requirement of Cellular ATP for Cholesterol Export in Cholesterol-Loaded Human Macrophages on Stimulation With LXR Agonists

To determine whether the ABCA1-dependent and independent mechanisms of cholesterol efflux necessitate energy in cholesterol-loaded human macrophages, THP-1 macrophages and HMDM were depleted in ATP (see Materials and Methods) and cholesterol efflux was then evaluated.

Reduction of intracellular ATP (~80%, data not shown) caused a profound alteration in ABCA1-mediated cholesterol efflux to apoAI from THP-1 macrophages and HMDM (Figure 5A, THP-1: −48%, P<0.0005 and HMDM: −37%, P<0.0005), clearly indicating that ABCA1 requires ATP for the export of free cholesterol from human macrophages to apoAI. By contrast, cellular cholesterol efflux to HDL particles was not affected by the depletion of cellular ATP in both THP-1 macrophages and HMDM (Figure 5B). However, in human macrophages stimulated by LXR/RXR agonists (22OH-C/9cRA), free cholesterol efflux to HDL was diminished by 21% in response to reduction in intracellular ATP levels; such reduction corresponded to a 46% to 49% decrement in the stimulatory effect of LXR agonists in THP-1 macrophages (P<0.05) and HMDM (P<0.005), respectively.

These data provide evidence that stimulation of cholesterol efflux to HDL by LXR agonists in cholesterol-loaded human macrophages necessitates intracellular ATP for generation of the necessary energy, whereas no energy is required under basal conditions. Additional experiments presented in detail in supplemental materials indicated that the Cla-1 receptor contributes to cholesterol efflux to HDL from human macrophages under basal conditions. Furthermore, these findings support a role for ABC transporters such as ABCA1 in mediating free cholesterol efflux to HDL in LXR-stimulated cholesterol-loaded human macrophages.

Discussion

The LXR transcription factors regulate expression of numerous genes involved in cholesterol absorption, in the degradation of cholesterol to bile acids, and in reverse cholesterol transport. Activation of LXRs enhances cholesterol export from lipid-loaded macrophages and blocks cholesterol absorption in intestinal cells, thereby suggesting that LXR expression is likely to be antiatherogenic. This hypothesis is supported by several studies using mice, genetically-deficient in LXR expression, and equally by activation of LXR in mice, using synthetic LXR agonists, in which the development of atherosclerosis is attenuated.9,10

In the present study, we have confirmed previous reports that expression of both ABCA1 and ABCG1 are strongly induced in human macrophages on LXR/RXR activation by both natural and synthetic LXR agonists.11 LXR agonists increased the expression of ABCA1 and ABCG1 significantly above levels induced by cholesterol loading. The induction of ABCG1 was particularly striking, with mRNA levels elevated 2- to 3-fold more than those of ABCA1 and Cla-1 in LXR-stimulated cholesterol-loaded macrophages, thereby reinforcing the hypothesis that ABCG1 represents a preferential target of LXR ligands in foam cells. In addition to the marked induction of ABCA1 and ABCG1 mRNA levels, we observed that expression of the Cla-1 receptor was induced 2-fold on stimulation with 22OH-C/9cRA, leading us to suggest that Cla-1 may also be a target for LXR-mediated upregulation in human macrophages. Several lines of evidence suggest that the expression of Cla-1 may be regulated by cholesterol loading in human macrophages. In agreement with our observation, Crestani et al reported that the expression of Cla-1 was induced by LXR in human macrophages.14 However, cellular cholesterol loading, as well as treatment of 6-day old human macrophages with 25-hydroxy-cholesterol, has been reported to decrease Cla-1 expression by others.15 Taken together with the observation that Cla-1 expression was upregulated by SREBP-2,16 these results clearly indicate...
that Cla-1 expression is regulated by the cholesterol content of human macrophages. Nevertheless, the degree of cholesterol loading as well as the nature of oxysterol species may differentially modulate Cla-1 expression.

In cholesterol-loaded human macrophages, LXR-mediated stimulation of ABCA1 and ABCG1 gene expression was accompanied by enhanced cholesterol efflux to both apoAI and HDL. The increased efflux to apoAI was roughly proportional to the LXR-induced changes in ABCA1 expression, consistent with the central role of ABCA1 in cholesterol export to this acceptor. In comparison, the increased efflux to HDL in response to LXR treatment was rather modest (1.4- to 2-fold) and much less than the matching increments in ABCG1 mRNA and protein. This finding suggested that the marked increase in ABCG1 expression on LXR treatment of cholesterol-loaded HMDM might not contribute proportionately to the enhanced cholesterol efflux to HDL induced by LXR agonists. In further agreement with this observation, silencing of ABCG1 expression in cholesterol-loaded human macrophages (both HMDM and THP-1) had no impact on cholesterol efflux from these cells to HDL. In contrast, the recent findings of Jakobsson et al17 indicate that LXR-dependent stimulation of cholesterol efflux to HDL from THP-1 macrophages results from the specific GPS2-mediated recruitment of LXR to the ABCG1 promoter/enhancer, as silencing of GPS2 ablated the effects of LXR on both ABCG1 expression and cholesterol efflux. However, this hypothesis was not tested directly by silencing ABCG1 expression. As our data clearly show that the suppression of ABCG1 expression in both THP-1 and primary human macrophages, either transiently or by stable knockdown, is without effect on LXR-stimulated cholesterol efflux, then GPS2/LXR-dependent cholesterol efflux must be independent of ABCG1 expression and presumably linked to one of the plethora of other biological activities involving GPS2. In agreement with our observations, Delvecchio et al18 recently reported that stimulation of cholesterol efflux to HDL by LXR agonists from human airway smooth muscle cells exclusively requires ABCA1 but not ABCG1. This result is in direct contrast with the situation in mouse macrophages, where deletion of ABCG1 significantly reduces cholesterol efflux from cells as we (this study and2) and others have previously reported.3,10 Discrepancies between the importance of ABCG1-dependent efflux from cholesterol-loaded mouse and human macrophages might reflect differences in the respective rates of transport of cholesterol between ABCG1-accessible and ABCG1-inaccessible subcellular locations. We also cannot completely exclude the possibility that the very low residual levels of ABCG1 in the various silenced human macrophages used in this study are still sufficient to contribute to HDL-mediated cholesterol efflux.

An alternative explanation is that the lack of any effect of ABCG1 knockdown on cholesterol export to HDL from cholesterol-loaded cells indicates that alternative pathways may largely contribute to this process. Our data indicate that cholesterol efflux to HDL from LXR-stimulated human macrophages requires intracellular ATP. ABCA1 has previously been shown to possess functional ATPase activity when reconstituted in liposomes,20 while mutations in the Walker domain A (nucleotide-binding domain) of ABCA1-impaired cholesterol efflux to apoAI without altering expression or trafficking of the ABCA1 protein.21 In our system, ABCA1-mediated cholesterol export to apoAI from HMDM was greatly reduced in response to depletion in cellular ATP. Taken together, these observations suggest that ABCA1 possesses functional ATPase activity and that this activity is required for cholesterol efflux to apoAI from human macrophages.

We therefore considered the possibility that ABCA1 also contributes to the ATP-dependent efflux of cholesterol to HDL. This notion was supported by experiments in which the normal stimulation of cholesterol efflux to HDL by LXR was completely ablated in HMDM from a subject with Tangier Disease, an observation consistent with our recent findings that cholesterol efflux to HDL from ABCA1-null mouse macrophages is greatly reduced.5 HDL is generally a poor acceptor for ABCA1-mediated cholesterol efflux, and in the present study we showed directly that the HDL preparation used did not support ABCA1-dependent cholesterol export. However it is possible that macrophages may have the capacity to remodel HDL, which may subsequently shed pre-β particles, the latter being more avid acceptors for cholesterol effluxed from ABCA1 than HDL. Such remodeling of HDL particles may depend directly on ABCA1 activity or, more likely, may reflect the activity of macrophage surface or secreted proteins, such as PLTP.22,23 Therefore it is possible that the increment in cholesterol efflux to HDL seen from both control and ABCG1-silenced HMDM and THP-1 macrophages may reflect ABCA1-dependent export to acceptors generated from HDL.

The Cla-1 receptor is robustly expressed in fully differentiated human macrophages (THP-1 and HMDM), and on the basis of the inhibitory effect of a receptor-blocking antibody it appears to contribute to the elimination of free cholesterol from cholesterol-loaded human macrophages. This hypothesis is consistent with our observation that depletion in cellular ATP did not alter cholesterol efflux to HDL in the absence of LXR induction, and favors a transport mechanism for which energy is not required. The generation of cell lines stably transfected with SR-BI/Cla-1 identified the ability of this receptor to promote cholesterol efflux to large cholesteryl ester-rich HDL particles.24 However its role in mediating cholesterol efflux from mouse macrophages is controversial,25,26 and studies of its role in HMDM are lacking. A role for macrophage Cla-1 in cholesterol efflux to HDL is consistent with previous studies indicating that Cla-1 is expressed in human atherosclerotic lesion macrophages.27 Cholesterol efflux to HDL from cholesterol-loaded human macrophages therefore appears to be at least partially mediated by a transport mechanism that does not necessitate energy, thus excluding the ATP-dependent transporters of the ABC family. Although we presently demonstrate that the Cla-1 receptor is implicated in this mechanism, our findings likely suggest that cholesterol efflux to HDL in human macrophages equally involves receptors/transporters, as distinct from ABC transporters, which have not been identified to date.
Accumulation of cholesterol in macrophages leads to foam cell formation, a critical step in the early stages of atherosclerosis. The present work provides new information on the mechanisms controlling cholesterol homeostasis in human macrophages, notably by the characterization of the respective contribution of Cla-1, ABCA1, and ABCG1 in the removal of free cholesterol to HDL. Indeed, our findings indicate that Cla-1 is involved in the elimination of the excess of free cholesterol in cholesterol-loaded human macrophages in a transport mechanism that does not necessitate energy. However in foam cells stimulated with LXRs agonists, in which cholesterol efflux to HDL occurs by an ATP-dependent transport mechanism, we demonstrated that ABCA1 is solely responsible for enhanced cholesterol efflux. Further studies are therefore needed not only to determine the role of ABCG1 in human macrophage biology but also in lipid metabolism in humans.

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Disclosures

None.

References

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Supplemental Materials & Methods.

Radioisotopic cholesterol efflux assays.

Human THP-1 macrophages and HMDM were cholesterol loaded for 24 h with 50 µg/mL [³H]cholesterol-labeled acetylated LDL (acLDL) (1 µCi/mL) in serum-free RPMI 1640 supplemented with 50 mM glucose, 2 mM glutamine, 0.2% BSA (RGGB) and 100 µg/ml penicillin / streptomycin. The labeling medium was removed and human macrophages were then equilibrated in RGGB for an additional 16-24 h period in the presence or in the absence of either 0.1 mM 8Br-cAMP (Sigma), 1 µM TO901317 (Sigma) or 4 µg/mL 22-hydroxy-cholesterol (22OH-C) and 1 µM 9-cis retinoic acid (9cRA) (Sigma). Cellular cholesterol efflux to 5 µg/mL lipid-free apoAI (Biodesign), or 15 µg/ml HDL-PL (density=1.063-1.21 g/mL) isolated from normolipidemic plasma by preparative ultracentrifugation, or 5% (v/v) normolipidemic plasma, was assayed in serum-free medium for a 4-hour chase period in the presence or absence of 0.1 mM 8Br-cAMP, 1 µM TO901317 or 4 µg/mL 22OH-C and 1 µM 9cRA. Finally culture media were harvested and cleared of cellular debris by a brief centrifugation. Cell radioactivity was determined by extraction in hexane-isopropanol (3:2), evaporation of the solvent and liquid scintillation counting (Wallac Trilux 1450 Microbeta). The percentage of cholesterol efflux was calculated as 100 x (medium cpm) / (medium cpm + cell cpm). ApoAI-specific cholesterol efflux was determined by subtracting non-specific cholesterol efflux occuring in apoAI-free medium.

The human Cla-1 receptor in each cell type was blocked in cholesterol efflux studies by incubation with 5 µg/mL of either a rabbit anti-Cla-1 blocking antibody
(IgG)², or a rabbit anti-Cla1 non-blocking antibody (IgG) as a control, for 30 min prior to the efflux period.

When required, depletion of cellular ATP was achieved as previously described ³ by incubation with 10 mM sodium azide in glucose free-RPMI media (Invitrogen) for 30 min prior to the efflux period; this agent blocks ATP synthesis by the mitochondrial respiratory chain. Cells were then washed and incubated in glucose free-RPMI media (Invitrogen) during the 4-hour chase period to prevent ATP synthesis via glycolysis.

Cellular cholesterol efflux from bone marrow-derived macrophages (BMDM) was performed as previously described ⁴.

**Mass cholesterol efflux assays.**

At the end of the efflux period, the media were removed and the cells lysed in 0.2 N NaOH. Cell and media samples were extracted and analyzed for free and esterified cholesterol mass by HLPC, as previously described ⁵. Cell proteins were measured using the BCA assay (Pierce). 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.

**Western blot analysis.**

Cells were lysed in RIPA buffer (10 mM TRIS, 1% (w/v) sodium cholate, 0.1% (v/v) Triton X-100, 0.1% (w/v) SDS, pH 7.4) containing protease inhibitors and separated on a 7% Tris-acetate gel (Invitrogen). Proteins (20 µg per lane) were transferred to nitrocellulose and membrane was blocked with 5% skim milk (in PBS/0.1% Tween) for 1h. ABCG1 and ABCA1 were detected using rabbit anti-hABCG1 (NB400-132;
Novus) and rabbit anti-hABCA1 (NB400-105; Novus), respectively, at 1:2500 and goat anti-rabbit/HRP (Dako) at 1:5000. Quantification of Western blots was performed using a Kodak Image Station 440 CF with Kodak 1D Image Analysis Software (Perkin Elmer).

RNA interference (RNAi)-mediated ABCG1 silencing using small interference (si)RNA.
ABCG1 knock-down (KD) macrophages were obtained by application of siRNA oligonucleotides (Dharmacon) targeted to the cDNA sequence of the human ABCG1 gene (Genebank# AY048757). The sequences of the siRNA were 5’-UCAUUGGCCUGUGUGACUUUU-3’ and 5’-P-AAGUACAGCAGGCCAUGAUU-3’, respectively. For cholesterol efflux experiments, THP-1 macrophages were grown in 24-well plates and transfected with 50 nM control siRNA (Dharmacon) or siRNA targeting human ABCG1 using lipofectamine RNAiMax (Invitrogen) according to the manufacturer's instructions. After a 24-h incubation at 37°C, ABCG1 KD and control macrophages were cholesterol loaded and labelled with $[^{3}H]$cholesterol for cholesterol efflux experiments as described above.

Stable knockdown of ABCG1 in THP-1 macrophages.
THP-1 cells in which ABCG1 expression was stably silenced (ABCG1 SKD THP-1) were generated by the stable transfection of THP-1 cells (ATCC TIB-202) using short hairpin RNA (shRNA) targeted to ABCG1. The shRNA sequence was designed using an on-line algorithm and was custom made (Invitrogen). The oligos were cloned into the pENTR-TOPO vector (Invitrogen) and subsequently cloned into the pBLOCK-it 6 DEST vector (Invitrogen), to create pDEST6-ABCG1. All constructs were verified by
sequencing. pDEST6-ABCG1 was prepared using an endotoxin-free maxiprep kit (Qiagen) and was transfected into THP-1 monocytes using the Amaza Nucleofector with Cell Line Kit V (Amaza). Positive clones were selected using blasticidin (Invitrogen). After expansion of these clones, ABCG1 silencing was assessed using RT-PCR and Western blot.

**Generation of CHO-hABCA1 cells.**

Human *Abca1* was cloned into the *Xba*I site of pcDNA™4/TO (Invitrogen) to create the plasmid pMG1 and transfected into T-REx™- CHO cells. A monoclonal stable tetracycline-inducible-ABCA1-CHO line was generated by propagating a single cell placed in a well of a 96-well CHO plate by serial dilution in the presence of 10 µg/mL Blasticidin S HCl. ABCA1 expression was induced by adding tetracycline (1µg/ml) to the medium.

**RNA extraction, reverse-transcription and quantitative-PCR.**

Human macrophages (THP-1 and HMDM) were cultured in 6-well plates and incubated in the presence or in the absence of 0.1 mM 8Br-cAMP, 1 µM TO901317 or 4 µg/mL 22OH-C and 1 µM 9cRA for 24 h at 37°C. Cells were then washed twice with cold PBS and total RNA was extracted using a NucleoSpin RNA II kit (Macherey-Nagel) according to the manufacturer's instructions. Then, 1500 ng of RNA was reverse transcribed with 75 ng of random hexamer using 200 units of M-MLV reverse transcriptase. An initial denaturation step for 5 mn at 68°C was followed by an elongation phase of 1 h at 42°C; the reaction was completed by a 5-mn incubation at 68°C.
Real time quantitative PCR was performed using a LightCycler LC480 (Roche). The reaction contained 2.5 ng of reverse transcribed total RNA, 150 pmol of forward and reverse primers and 5µl of Master Mix SYBR-Green, in a final volume of 10µl. Samples underwent the standard PCR protocol. Crossing point (CP) values for genes of interest were normalized to housekeeping genes (human delta-aminolevulinate synthase and human alpha-tubulin). Expression data were based on the crossing points calculated with the software for LightCycler data analysis and corrected for PCR efficiencies of the target and the reference gene. When indicated, data were expressed as a fold change in mRNA expression relative to control values.

**Statistical analyses.**

Data are shown as mean ± SEM. Experiments were performed in triplicate and values correspond to the mean from at least three independent experiments. Comparisons of 2 groups were performed by a 2-tailed Student’s $t$ test and comparisons of 3 or more groups were performed by ANOVA with Newman–Keuls post-test. All statistical analyses were performed using Prism software from GraphPad (San Diego, CA, USA).
Supplemental Results.

*ABCA1 and ABCG1 mRNA levels are highly stimulated by LXR agonists in human macrophages.*

To explore the relative contribution of the ABCA1, ABCG1 and Cla-1 pathways to cholesterol efflux in human macrophages and foam cells, the expression of these transporters was examined in human THP-1 macrophages differentiated with PMA and in human monocyte-derived macrophages (HMDM) by quantification of mRNA levels. In macrophages, excess cholesterol leads to the formation of oxysterols, which are abundant in macrophages of the atherosclerotic plaque. Hydroxycholesterol (HC) derivatives stimulate both ABCG1 and ABCA1 gene expression in both mouse and human macrophages by activating the nuclear receptor LXR.

We first analyzed and compared the capacity of natural LXR/RXR agonists (22OH-C/9cRA), synthetic LXR agonist TO901317 and 8Br-cAMP, (this latter has been demonstrated to strongly stimulate ABCA1 gene expression in the mouse), to stimulate the expression of ABCA1, ABCG1 and Cla-1 genes in human macrophages (THP-1 and HMDM, Figure I). Incubation with 8Br-cAMP allowed a modest ~2-fold induction of ABCA1 (THP-1: 1.3-fold and HMDM: 2.6-fold, p<0.0005) and ABCG1 mRNA levels (THP-1: 1.5-fold, p<0.05 and HMDM: 3.9-fold, p<0.0005), but was without effect on Cla-1. ABCA1 gene expression was equally stimulated in both THP-1 macrophages and HMDM by either TO901317 (5.8-fold and 9.6-fold, p<0.0005, respectively) or 22OH-C/9cRA (15.6-fold and 18.1-fold, p<0.0005, respectively). More strikingly, ABCG1 mRNA levels were increased 3.2-fold by TO901317 (p<0.0005) and 6.8-fold by 22OH-C/9cRA (p<0.0005) in THP-1 macrophages, attaining 28.6-fold (p<0.0005) and 44.6-fold (p<0.0005) elevations,
respectively, in HMDM. Although LXR agonists exerted a profound effect on both ABCA1 and ABCG1 mRNA levels, 22OH-C/9cRA but not TO901317 was responsible for a modest ~2-fold elevation in Cla-1 mRNA levels (THP-1: 2.2-fold, p<0.005 and HMDM: 1.9-fold, p<0.0005). The overall effects of LXR agonists and 8Br-cAMP on ABCA1, ABCG1 and Cla-1 mRNA levels in THP-1 macrophages and HMDM was summarized in Panel C of Figure I. Stimulation of ABCA1 and ABCG1 expression by LXR agonists detected by analysis of mRNA levels was then confirmed semi-quantitatively at the protein level by Western blots (data not shown).

**Effect of HDL concentration on cholesterol efflux from control and ABCG1-silenced macrophages.**

We routinely use HDL at 15 µg PL/mL to stimulate ABCG1-dependent cholesterol efflux, as we have previously shown that this concentration is sufficient for the maximal rate of efflux mediated by this transporter. Further increasing the HDL concentration only increases non-specific, ABCG1-independent, cholesterol export. However, to exclude the possibility that ABCG1 may contribute to cholesterol export from human macrophages at higher HDL acceptor concentrations, we have measured cholesterol efflux to higher concentrations of HDL from Control (Ctrl) and ABCG1 Knockdown (KD) THP-1 macrophages. The data indicate that the silencing of ABCG1 expression by 90% (Supplemental Figure IIA-B) did not alter cholesterol efflux to HDL from THP-1 macrophages (±LXR ligand) at HDL concentrations up to 100 µg/mL HDL-PL (Supplemental Figure IIC). Similar experiments comparing control and ABCG1-stably knocked down (ABCG1 SKD) THP-1 cells gave the same result (data not shown).
Absence of cholesterol acceptors that can promote cholesterol efflux via ABCA1 in the HDL preparation.

In order to rule out the possibility that the HDL preparation used in our study contains species (such as lipid-poor ApoAI) that can promote cholesterol efflux via ABCA1, we tested our HDL preparation in a CHO-hABCA1 cell system in which the ABCA1 expression was induced by the addition of 1 µg/mL tetracycline (Supplemental Figure IIIB). Whereas cholesterol efflux to ApoAI was strongly increased by the induction of ABCA1 expression (Supplemental Figure IIIC), no difference was observed when cholesterol efflux to our HDL preparation was measured (Supplemental Figure IIID). Our data therefore indicate that the knockdown of ABCG1 activity in human macrophages was not compensated by an increased ABCA1-mediated cholesterol efflux due to the presence of cholesterol acceptors in the HDL preparation acting preferentially with ABCA1.

Effect of cholesterol loading on cholesterol efflux from control and ABCG1-silenced macrophages.

In this study, human macrophages were loaded with acetylated LDL (acLDL), which is a well-understood and validated procedure to lead to the accumulation of cholesteryl ester (CE) as observed in foam cells. However; in order to rule out the possibility that the contribution of ABCG1 in cholesterol efflux from human macrophages is dependent of the cholesterol loading condition, we have tested alternative cholesterol loading procedures including oxidized LDL (50 µg/mL), cholesterol-cyclodextrin complexes (20 µg/mL) and triglyceride-rich lipoproteins (100 µg/mL) (Figure IV). All of these loading conditions generated the same observation
we reported with acLDL, i.e. efflux to HDL was identical in control and ABCG1-silenced THP-1 macrophages.

**Cellular cholesterol efflux to HDL in cholesterol-loaded human macrophages requires the Cla-1 receptor.**

The robust expression of the Cla-1 receptor in THP-1 macrophages and HMDM (Figure 1), in addition to data indicating that mechanisms of free cholesterol efflux to HDL do not require cellular ATP in these cells under basal conditions (Figure 5), support a role for this receptor in mechanisms of cholesterol export in human macrophages. To test this possibility, cholesterol efflux analysis was evaluated in cholesterol-loaded human macrophages using a specific anti-Cla-1 blocking antibody which has been demonstrated to suppress Cla-1 activity (see Materials and Methods).

As compared to a non-blocking Cla-1 antibody, the incubation of acLDL-loaded human macrophages with the blocking Cla-1 antibody, at a concentration that efficiently abrogates Cla-1 activity, was accompanied by a 20% reduction ($p<0.0001$) of $[^3H]$cholesterol efflux to HDL in both THP-1 macrophage and HMDM (Figure V). This finding indicates that the Cla-1 receptor contributes to human macrophage cholesterol homeostasis via its role in mediating free cholesterol efflux to HDL.
References.


Supplemental Figures.

**Figure I.** ABCA1 and ABCG1 mRNA levels are highly stimulated by LXR agonists in human macrophages. A, THP-1 macrophages and B, HMDM were incubated in the presence or in the absence of 0.1 mM 8Br-cAMP, 1 µM TO901317 or 4 µg/mL 22-hydroxycholesterol (22OH-C) and 1 µM 9-cis retinoic acid (9cRA) for 24 h. Total RNA was then extracted, quantified by Real time quantitative PCR and normalized to both human delta-aminolevulinate synthase and human alpha-tubulin housekeeping genes. Values are the mean ± SEM of 9 independent experiments and expressed as a fold change relative to cells incubated in the absence of agonists. *p<0.05, **p<0.005 and ***p<0.0005 compared with cells not treated with agonist. C, Summary of the effect of each agonist on ABCA1, ABCG1 and Cla-1 mRNA levels in THP-1 macrophages and HMDM. +, induction; ↔, no change.

**Figure II.** Stimulation of cholesterol efflux to HDL by LXR agonist does not require the transporter ABCG1 in cholesterol-loaded THP-1 macrophages. THP-1 macrophages were transfected with control siRNA (Ctrl) or siRNA targeting human ABCG1 (ABCG1 KD) and cholesterol loaded with [3H]cholesterol-labeled acetylated LDL (acLDL) for a 24h-period. Cells were stimulated with or without 22-hydroxycholesterol (22OH-C) and 9-cis retinoic acid (9cRA) for 24 h prior to analysis. A, Total ABCG1 protein was assessed by Western blot analysis. B, The efficiency of the ABCG1 knockdown was assessed by quantification of mRNA (-84%) and protein (-90%) levels. ABCG1 mRNA was normalised to housekeeping genes (δ-aminolevulinate synthase and human α-tubulin) and ABCG1 protein to matching annexin1 levels. C, Cholesterol efflux to increasing amounts of HDL (0, 15, 25, 50
and 100 µg/mL HDL-PL) was assayed for a 4-hour chase period in the presence or absence of 22OH-C/9cRA. Values are mean ± SD.

**Figure III.** The absence of effect on cholesterol efflux to HDL in response to the ABCG1 knockdown does not result to a compensatory increased ABCA1-mediated cholesterol efflux. ABCA1 protein expression was assessed by Western blot analysis in cholesterol-loaded Ctrl and ABCG1 KD HMDM stimulated or not with 22OH-C/9cRA (A) and in a CHO-hABCA1 inducible cell system (+/- 1 µg/mL tetracycline (Tet)) (B). Cholesterol efflux to lipid-free ApoAI (C) or HDL (D) from CHO-hABCA1 cells treated in the presence (+ABCA1) or absence (-ABCA1) of 1 µg/mL tetracycline. Values are mean ± SD.

**Figure IV.** The contribution of ABCG1 to cholesterol efflux from human macrophages is not dependent of the cholesterol loading procedure. THP-1 macrophages transiently transfected with control siRNA (Ctrl) or siRNA targeting ABCG1 (ABCG1 KD) (A, C); or THP-1 macrophages stably transfected with shRNA targeting human ABCG1 (ABCG1 SKD) (B, D) were cholesterol-loaded with 20 µg/mL cholesterol-cyclodextrin complexes (CD) for 6 hours (A-B) or with either 50 µg/mL oxidized LDL (oxLDL) (C) or 100 µg/mL triglyceride-rich lipoproteins (TGRL) (D) for a 24h-period. Cholesterol-loaded cells were stimulated with or without 22-hydroxycholesterol (22OH-C) and 9-cis retinoic acid (9cRA) for 24 h and cholesterol efflux to HDL was assayed for a 4-hour chase period in the presence or absence of 22OH-C/9cRA. Values are mean ± SD.
**Figure V.** Reduction of cholesterol efflux to HDL by inhibition of the Cla-1 receptor in cholesterol-loaded human macrophages. Cholesterol efflux to HDL in cholesterol-loaded THP-1 macrophages (A) and HMDM (B) in the presence of either a blocking (B) or a non-blocking (NB) anti-Cla-1 antibody. Values are means ± SEM and expressed as relative to cells incubated with NB antibody. *p<0.0005 versus cells incubated with NB antibody.*
Figure I

A

THP-1

Relative mRNA Expression (Fold Induction)

8Br-cAMP  -  +  -  -
TO901317  -  -  +  -
22OH-C/9cRA  -  -  -  +

B

HMDM

Relative mRNA Expression (Fold Induction)

8Br-cAMP  -  +  -  -
TO901317  -  -  +  -
22OH-C/9cRA  -  -  -  +

C

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Figure II

A

THP-1

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22OH-C/9cRA

B

![Graph showing % of ABCG1 mRNA and Protein](image)

C

![Graph showing [3H]Cholesterol Efflux to HDL](image)
Figure III

A

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B

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C

![Graph showing cholesterol efflux to ApoAI](image)

D

![Graph showing cholesterol efflux to HDL](image)
Figure IV

A  CD

22OH-C/9cRA

\[^{3}H\]Cholesterol Efflux to HDL (% of total)

-  +

\[\text{ABCG1 KD} \quad \text{Ctrl}\]

B  CD

22OH-C/9cRA

\[^{3}H\]Cholesterol Efflux to HDL (% of total)

-  +

\[\text{ABCG1 SKD} \quad \text{Ctrl}\]

C  oxDLDL

22OH-C/9cRA

\[^{3}H\]Cholesterol Efflux to HDL (% of total)

-  +

\[\text{ABCG1 KD} \quad \text{Ctrl}\]

D  TGRL

22OH-C/9cRA

\[^{3}H\]Cholesterol Efflux to HDL (% of total)

-  +

\[\text{ABCG1 SKD} \quad \text{Ctrl}\]
Figure V

A

THP-1

Relative $[^3]H$ Cholesterol Efflux to HDL

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B

HMDM

Relative $[^3]H$ Cholesterol Efflux to HDL

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