Deletion of ABCA1 and ABCG1 Impairs Macrophage Migration Because of Increased Rac1 Signaling

Tamara A. Pagler, Mi Wang, Mousumi Mondal, Andrew J. Murphy, Marit Westerterp, Kathryn J. Moore, Frederick R. Maxfield, Alan R. Tall

Rationale: Reduced plasma cholesterol and increased high-density lipoprotein (HDL) levels promote regression of atherosclerosis, in a process characterized by lipid unloading and emigration of macrophages from lesions. In contrast free cholesterol loading of macrophages leads to imbalanced Rac1/Rho activities and impaired chemotaxis.

Objective: To study the role of HDL and the ATP-binding cassette transporters ABCA1 and ABCG1 in modulating the chemotaxis of macrophages.

Methods and Results: Abca1<sup>−/−</sup>/Abcg1<sup>−/−</sup> mouse macrophages displayed profoundly impaired chemotaxis both in a Transwell chamber assay and in the peritoneal cavity of wild-type (WT) mice. HDL reversed impaired chemotaxis in free cholesterol-loaded WT macrophages but was without effect in Abca1<sup>−/−</sup>/Abcg1<sup>−/−</sup> cells, whereas cyclodextrin was effective in both. Abca1<sup>−/−</sup>/Abcg1<sup>−/−</sup> macrophages had markedly increased Rac1 activity and increased association of Rac1 with the plasma membrane (PM). Their defective chemotaxis was reversed by a Rac1 inhibitor. To gain a better understanding of the role of transporters in PM cholesterol movement, we measured transbilayer PM sterol distribution. In WT macrophages, the majority of cholesterol was located on the inner leaflet, whereas on upregulation of transporters by liver X receptor activation, PM sterol was shifted to the outer leaflet, where it could be removed by HDL. Abca1<sup>−/−</sup>/Abcg1<sup>−/−</sup> macrophages showed increased PM sterol content and defective redistribution of sterol to the outer leaflet.

Conclusions: Deletion of ABCA1 and ABCG1 causes an increased cholesterol content on the inner leaflet of the PM, associated with increased Rac1 PM localization, activation, and impairment of migration. ABCA1 and ABCG1 facilitate macrophage chemotaxis by promoting PM transbilayer cholesterol movement and may contribute to the ability of HDL to promote regression of atherosclerosis. (Circ Res. 2011;108:194-200.)

Key Words: ABCA1 ■ ABCG1 ■ migration ■ Rac1 ■ HDL

Raising high-density lipoprotein (HDL) levels has emerged as a potential therapeutic intervention in atherosclerotic cardiovascular disease. Clinical trials of reconstituted human HDL infusions in patients with established cardiovascular disease demonstrated a significant reduction in atheroma volume suggesting active regression.1,2 Regression of atherosclerosis by lowering cholesterol or increasing HDL has also been shown in mouse models.3–5 Using a surgical approach, aortic segments with established lesions from hypercholesterolemic Apoe<sup>−/−</sup> mice were transplanted into wild-type (WT) and Apoe<sup>−/−</sup> recipients.3 Transplantation of lesions into WT recipients resulted in depletion of foam cells and a marked regression of atherosclerotic plaques, whereas in Apoe<sup>−/−</sup> recipients with high cholesterol and low HDL levels, further lesion progression was seen.4,6 The reduction of plaque area was correlated with emigration of macrophages to regional and systemic lymph nodes. Laser capture microscopy analysis of foam cells from regressing lesions indicated up regulation of liver X receptor (LXR) and ABCA1.7 Furthermore, in Apoe<sup>−/−</sup>/Apoa1<sup>Tg</sup> recipients with elevated HDL levels lipid unloading and depletion of macrophage foam cells from plaques were observed despite high cholesterol levels.5 This suggests that increased HDL perhaps acting in conjunction with ABC transporters promotes cholesterol efflux and stimulates emigration of macrophages from plaques.

In contrast to the beneficial effects of cholesterol efflux, plasma membrane (PM) free cholesterol loading caused impaired chemotaxis of macrophages, associated with increased levels of Rac-GTP and reduced RhoA activity.8,9 A number of molecular events need to be integrated to allow a cell to become motile. Small Rho-GTPases play a pivotal role in actin filament organization and therefore in the coordination of cell migration.10 Rho-GTPases function as molecular switches in which the exchange of GDP for GTP triggers a conformational change that allows binding and activation of downstream effectors to direct cytoskeleton remodeling. To be in their active state, small
GTPases translocate to the PM,\textsuperscript{11} which is facilitated by post-translational isoprenylation and fatty acylation.

The ATP-binding cassette transporters ABCA1 and ABCG1 promote cholesterol efflux to apolipoprotein (apo)A1 and HDL, respectively.\textsuperscript{12,13} Hypercholesterolemic \textit{Ldlr}^−/− mice transplanted with \textit{Abca1}^−/−\textit{Abcg1}^−/− bone marrow show significantly accelerated atherosclerosis and foam cell accumulation in various tissues.\textsuperscript{14,15} Of particular interest is the ability of ABCA1 and ABCG1 to modulate PM cholesterol levels and lipid organization.\textsuperscript{16} PM staining with the ganglioside-binding cholera toxin B has suggested an increase of liquid ordered membrane domains (lipid rafts) in \textit{Abca1}^−/− or \textit{Abcg1}^−/− macrophages and an even more pronounced increase in \textit{Abca1}^−/−\textit{Abcg1}^−/− macrophages.\textsuperscript{17−21} In this study, we initially explored the hypothesis that HDL and ABC transporters might act to facilitate chemotaxis of cholesterol-loaded macrophages. We further investigated the relationship of impaired chemotaxis of \textit{Abca1}^−/−\textit{Abcg1}^−/− cells to Rac1 activation, and related this to changes in PM lipid organization, using a novel fluorescence technique to monitor transbilayer sterol distribution.\textsuperscript{22}

**Methods**

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

**Animals**

WT and \textit{Abca1}^−/−\textit{Abcg1}^−/− littermates in a mixed C57BL/6×DBA background, as described previously, were used.\textsuperscript{14} All mice were housed at Columbia University Medical Center according to animal welfare guidelines. Animals had ad libitum access to food and water.

**Sterol Transbilayer Distribution**

As described previously\textsuperscript{22} cells were trace-labeled with cholestatrienol–methyl-β-cyclodextrin (CTL-MβCD) complexes (0.5 mmol/L) for 1 minute at 37°C, washed, and then incubated for 10 minutes at 37°C to reach a steady-state distribution of CTL. Fluorescence images were taken before and after addition of the membrane impermeable fluorescence quencher 2,4,6-trinitrobenzenesulphonic acid (TNBS) at 10 mmol/L. The PM associated CTL-fluorescence was measured before and after quenching and depicted as percentage quenching.

**Results**

\textit{Abca1}^−/−\textit{Abcg1}^−/− Macrophages Show Impaired Migratory Responses

The ability of WT and \textit{Abca1}^−/−\textit{Abcg1}^−/− macrophages (thioglycollate-elicited) to migrate toward the strong chemoattractant C5a was determined using a Transwell chamber. Chemotaxis was markedly reduced for \textit{Abca1}^−/−\textit{Abcg1}^−/− compared to WT macrophages (Figure 1). Similarly, migration of \textit{Abca1}^−/−\textit{Abcg1}^−/− macrophages was also impaired in response to MCP-1 and CCL21, a ligand of CCR7 that has been implicated in regression of lesions (Online Figure I). Therefore, downstream signaling and cellular responses for various chemokine receptors including atherorelevant CCR2 and CCR7 receptors are likely similar and thus we continued to use C5a for our studies.\textsuperscript{23,24} Although combined deficiency of ABCA1 and ABCG1 dramatically reduced migration of macrophages, knock out of ABCG1 or ABCA1 alone did not significantly affect the migration of macrophages (Online Figure II, A and B), consistent with previous data indicating overlapping roles and mutual compensation by these two transporters.\textsuperscript{14}

Increased levels of membrane free cholesterol led to the abrogation of the migration responses in macrophages.\textsuperscript{9} Therefore, we treated WT and \textit{Abca1}^−/−\textit{Abcg1}^−/− macrophages with cyclodextrin complexed cholesterol and then carried out the Transwell migration assay (Figure 1). As reported,\textsuperscript{9} there was an almost complete abolition of chemotaxis in WT macrophages with no further impairment in \textit{Abca1}^−/−\textit{Abcg1}^−/− macrophage migration. The cholesterol mediated impairment of migration was partly reversed by pretreatment with 100 μg/mL HDL2 in WT but not in \textit{Abca1}^−/−\textit{Abcg1}^−/− macrophages indicating the importance of ABCA1 and ABCG1 mediated cholesterol efflux in the restoration of migratory function. To further test if

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**Non-standard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ABC</td>
<td>ATP-binding cassette transporter</td>
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<tr>
<td>acLDL</td>
<td>acetylated low-density lipoprotein</td>
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<tr>
<td>apo</td>
<td>apolipoprotein</td>
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<tr>
<td>CTL</td>
<td>cholestatrienol</td>
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<td>ER</td>
<td>endoplasmic reticulum</td>
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<td>HDL</td>
<td>high-density lipoprotein</td>
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<td>LDL</td>
<td>low-density lipoprotein</td>
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<tr>
<td>LXR</td>
<td>liver X receptor</td>
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<tr>
<td>MβCD-C</td>
<td>methyl-β-cyclodextrin-cholesterol</td>
</tr>
<tr>
<td>OxLDL</td>
<td>oxidized low-density lipoprotein</td>
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<tr>
<td>PM</td>
<td>plasma membrane</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>TNBS</td>
<td>2,4,6-trinitrobenzenesulfonic acid</td>
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<td>WT</td>
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**Figure 1.** \textit{Abca1}^−/−\textit{Abcg1}^−/− macrophages show impaired Transwell migration not restorable by HDL. Thiglycollate-elicited macrophages from WT or \textit{Abca1}^−/−\textit{Abcg1}^−/− mice were seeded on Transwell filters, and the migratory capacity was tested by stimulation with 10 nmol/L C5a for 2 hours. Where indicated, cells were treated with 5 mmol/L MβCD-C (8:1) with and without 100 μg/mL HDL2 for 30 minutes before subjecting cells to migration. Migrated macrophages were quantified after fixation and DAPI staining. Results were normalized to the number of untreated WT macrophages that migrated. The results shown are means ± SEM of 5 independent experiments performed in triplicates. **P<0.001 vs WT−C5a or WT+C5a; **P<0.01 vs WT+C5a; #P<0.01 vs WT cholesterol-loaded; §§§P<0.001 vs WT+C5a; §§P<0.01 vs WT cholesterol-loaded and HDL-treated.
Increased levels of PM cholesterol are the direct cause of impaired migration in Abca1\(^{-/-}\)Abcg1\(^{-/-}\) macrophages. We treated control or cholesterol-loaded WT and Abca1\(^{-/-}\)Abcg1\(^{-/-}\) macrophages with cholesterol-poor MβCD before evaluation of migration behavior. Reduction of PM cholesterol completely restored the ability of Abca1\(^{-/-}\)Abcg1\(^{-/-}\) macrophages to migrate, with or without prior cholesterol loading, and reversed the effect of prior cholesterol loading in WT macrophages (Online Figure III).

To study the migratory behavior of Abca1\(^{-/-}\)Abcg1\(^{-/-}\) macrophages in vivo, we used a bead labeling assay. First, equal amounts of red and green labeled WT thioglycollate-elicited macrophages were injected into the peritoneal cavity of thioglycollate pretreated WT recipients. 12 hours after injection the amount of retained labeled macrophages was analyzed (Figure 2A). Similar amounts of WT red and green labeled macrophages were found indicating that the bead label does not influence macrophage behavior. Next, equal amounts of WT (red) and Abca1\(^{-/-}\)Abcg1\(^{-/-}\) (green) labeled macrophages were injected into the peritoneum of WT recipient mice (Figure 2B). Twelve hours after injection, 7-fold more Abca1\(^{-/-}\)Abcg1\(^{-/-}\) macrophages remained in the peritoneum of WT recipient mice. Twelve hours after injection, 7-fold more macrophages were found indicating that the bead label does not influence macrophage behavior. Next, equal amounts of WT (red) and Abca1\(^{-/-}\)Abcg1\(^{-/-}\) (green) labeled macrophages were injected into the peritoneum of WT recipient mice (Figure 2B). Twelve hours after injection, 7-fold more Abca1\(^{-/-}\)Abcg1\(^{-/-}\) macrophages remained in the peritoneal cavity, indicating a profound defect in emigration compared to WT macrophages.

**Increased Actin Polymerization, Membrane Ruffling, and Cell Spreading of Abca1\(^{-/-}\)Abcg1\(^{-/-}\) Macrophages**

Cell motility is largely coordinated by the actin cytoskeleton, a key mediator of cell polarization and the directed migration of macrophages. Freshly isolated peritoneal macrophages were stained for F-actin to assess morphological and cytoskeletal changes. Abca1\(^{-/-}\)Abcg1\(^{-/-}\) macrophages exhibited increased ruffling of the cell membrane indicating F-actin polymerization (Online Figure IV). Extension of the cell membrane (lamellipodia formation) was observed surrounding cells (arrowheads). Increased ruffling at the dorsal side of the Abca1\(^{-/-}\)Abcg1\(^{-/-}\) macrophages (arrows) was also seen. Quantification of the cell adhesion area of WT and Abca1\(^{-/-}\)Abcg1\(^{-/-}\) macrophages furthermore showed that Abca1\(^{-/-}\)Abcg1\(^{-/-}\) macrophages are more spread out, with a 2-fold increase in the average cell area (Figure 3). Overall Abca1\(^{-/-}\)Abcg1\(^{-/-}\) macrophages show increased circumferential lamellipodia formation.

**Impaired Migration in Abca1\(^{-/-}\)Abcg1\(^{-/-}\) Macrophages Is Attributable to Increased Rac1-GTP Levels**

Rho GTPases such as Rac, RhoA, and Cdc42 are well known to play a crucial role in cell migration. Rac1 especially has been linked to membrane ruffle/lamellipodia formation with the highest concentrations of active Rac located at the leading edge...
of motile cells. Freshly isolated Abca1–/– Abcg1–/– macrophages showed a robust 1.4 fold increase in Rac1 activity as shown by Rac1-GTP levels (Figure 4A). Abca1–/– or Abcg1–/– macrophages did not have significantly altered Rac1-GTP levels (Online Figure V, A and B), consistent with their normal migratory ability. To further confirm a link between impaired migration of Abca1–/– Abcg1–/– macrophages and increased Rac1 activation we used an inhibitor to reduce Rac activation in a Transwell migration assay. The Rac inhibitor NSC23766 specifically and reversibly targets Rac1 GDP/GTP exchange activity thereby lowering Rac-GTP levels. Reduction of Rac1-GTP levels slightly increased migration in WT macrophages and restored migration in Abca1–/– Abcg1–/– macrophages to WT levels (Figure 4B). As previous reports indicate a reduction of RhoA-GTP on increased activity of Rac1 we measured RhoA-GTP levels by ELISA (Online Figure VI). We did not find any significant RhoA-GTP on increased activity of Rac1 we measured RhoA-GTP levels (Figure 4B). As previous reports indicate a reduction of phagocytosis were treated with the Rac inhibitor NSC23766 (100 nM) before cell lysates were analyzed for Rac1-GTP levels using a commercially available ELISA kit (A). Thioglycollate-elicited macrophages from WT and Abca1–/– Abcg1–/– mice were subjected to 2 hours of Transwell migration as described in Figure 1. Where indicated, macrophages were treated with the Rac inhibitor NSC23766 (100 µmol/L) during the time of migration. The results shown are means±SEM of 3 independent experiments performed in triplicates. **P<0.01 vs WT (A); ***P<0.001 vs WT–C5a; #P<0.05 vs WT+C5a; ###P<0.001 vs Abca1–/– Abcg1–/–+C5a; §§§P<0.001 vs WT+C5a (B).

**Abca1–/–Abcg1–/– Macrophages Show Increased PM Sterol and an Inability to Move Sterol From the Inner to the Outer Leaflet of the PM Bilayer**

PM cholesterol levels have an important role in the regulation of cell migration. To directly investigate whether excess levels of PM cholesterol are the underlying cause of the migratory dysfunction in Abca1–/– Abcg1–/– macrophages PM cholesterol levels were measured in freshly isolated WT and Abca1–/– Abcg1–/– peritoneal macrophages using filipin staining. Abca1–/– Abcg1–/– macrophages showed a 1.4-fold increase of filipin staining, suggesting increased free cholesterol in the PM (Figure 6), in agreement with previous data showing an increase

**Figure 4. Abca1–/–Abcg1–/– macrophages exhibit markedly increased Rac1 activation.** Thioglycollate-elicited macrophages from WT and Abca1–/– Abcg1–/– mice were serum-depleted for 24 hours before cell lysates were analyzed for Rho1-GTP levels using a commercially available ELISA kit (A). Thioglycollate-elicited macrophages from WT and Abca1–/– Abcg1–/– mice were subjected to 2 hours of Transwell migration as described in Figure 1. Where indicated, macrophages were treated with the Rac inhibitor NSC23766 (100 µmol/L) during the time of migration. The results shown are means±SEM of 3 independent experiments performed in triplicates. **P<0.01 vs WT (A); ***P<0.001 vs WT–C5a; #P<0.05 vs WT+C5a; ###P<0.001 vs Abca1–/– Abcg1–/–+C5a; §§§P<0.001 vs WT+C5a (B).

**Figure 5. Increased Rac localization to the PM of Abca1–/– Abcg1–/– macrophages.** Thioglycollate-elicited macrophages from WT and Abca1–/– Abcg1–/– macrophages were plated overnight in regular growth media and subsequently serum-starved for 48 hours. Cells were then lyzed in hypertonic buffer and subjected to ultracentrifugation to separate the PM from the cytosol fraction. An immunoblot for Rac1,2,3 was performed. Representative blot of Rac positive signal at 20 kDa is shown (A). Band densities normalized to β-actin are shown in a bar graph (B). Data represent means±SEM of a total of 4 to 5 mice per group in 2 independent experiments. *P<0.05 vs WT.

**Figure 6. Increased PM Sterol and an Inability to Move Sterol From the Inner to the Outer Leaflet of the PM Bilayer**

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transmission electron microscopy shows that Abca1 and Abcg1 double deficient macrophages were unable to move sterol from the inner to the outer PM leaflet. Transbilayer PM cholesterol distribution was measured in macrophages from WT and Abca1−/− Abcg1−/− mice. Macrophages were trace-labeled with the fluorescent cholesterol analog MβCD-cholestatrienol for 1 minute, washed, and equilibrated for 10 minutes at 37°C. Subsequently, images were taken by focusing at the PM before the addition of the cell membrane nonpermeable quenching reagent TNBS (A). Distribution of CTL to PM, ER, and lipid droplets was observed (B). TNBS at 10 mM was added on the microscope stage, and images of the same field were taken within 1 minute; note the decrease of fluorescence intensity after TNBS addition within the yellow circles (B). The quenching of exofacial incorporated CTL by TNBS was measured at multiple peripheral areas along the PM of each cell and depicted as percentage quenching (C). Where indicated, cells were treated with acLDL (50 μg/mL) and the LXRs compound T0-901317 (5 μM/L). To test the reversibility of this treatment, 100 μg/mL HDL was added in a separate group. A total of 30 to 100 cells for each treatment condition were analyzed of 3 independent experiments. Data are shown as means±SEM. ***P<0.001 vs basal WT; #P<0.05 vs acLDL+T0 WT; §§§P<0.001 vs WT acLDL+T0.

Discussion

Herein, we demonstrate the importance of the cholesterol transporters ABCA1 and ABCG1 in macrophage chemotaxis. Increased PM cholesterol content and an inability to move sterol across the PM bilayer in Abca1−/− Abcg1−/− macrophages are associated with increased PM localization and activation of Rac1, enhanced PM ruffling, cell spreading, and abrogation of migration toward various chemotactic stimuli including C5a, MCP-1, and CCL21. Abca1−/− Abcg1−/− myeloid cells show multiple abnormalities in cell motility, proliferation in response to growth factors, and viability that likely contribute to accelerated atherosclerosis in Abca1−/− Abcg1−/− mice. The present study suggests that a fundamental underlying cellular mechanism may be altered transbilayer sterol distribution, formation of cholesterol-rich liquid ordered domains on the PM, and excessive activation of small GTPases such as Rac1 and Ras.

Although the detailed molecular mechanisms and sites of activity of ABCA1 and ABCG1 remain poorly understood, our findings show for the first time that both transporters promote the movement of sterol across the PM bilayer, where it becomes accessible to HDL for removal. Moreover, this activity is observed in the absence of HDL (Figure 7C) and probably explains the finding that transporter deficiency leads to suppression of endoplasmic reticulum (ER) sterol-regulated genes, because depletion of the inner leaflet of the PM of sterol by transporter activity should lead to rapid diffusional replenishment from other organelles such as ER.

Abca1−/− Abcg1−/− macrophages showed profoundly impaired chemotaxis both in a Transwell chamber assay and in vivo. During migration cells undergo the so-called cell motility cycle starting with the polarization of the cell and resulting in extensions of lamellipodia at the leading edge. This is followed by the formation of focal adhesions to the underlying substrate and ends in the contraction and detachment at the rear and the movement of the cell. These multiple steps depend on coordinated regulation by the Rho Family of small GTPases. We found an increase in the active form of the small GTPase Rac1 in double deficient macrophages. The role of increased Rac1-GTP for the observed migration defect in Abca1−/− Abcg1−/− macrophages was confirmed by the ability to restore migration by reducing Rac1-GTP levels. Active Rac at the leading edge of lamellipodia is thought to mediate actin polymerization, producing lamellipodial extension and forward cell movement. Strong enhancement of Rac activity was shown to disrupt cell migration with a substantial decrease in velocity. It seems that a tight regulation must be kept on the level of Rac activity and that substantial increase in Rac activity leads to increased attachment with the substratum and/or the loss of correct polarization keeping the cell fixed to the substratum and immobile.
Given that the total cholesterol content in the PM of Abca1−/− Abcg1−/− macrophages is increased, our findings indicate an increase in the sterol content and very likely an increase in formation of liquid ordered domains on the inner leaflet of the PM in Abca1−/− Abcg1−/− cells. Rac activation is accompanied by its translocation to the PM. The capacity of small GTPases like Rac to cycle on and off membranes is thought to be integral to their biological activity as they stay in their active GTP state as long as they are associated with the membrane. Consistent with this we found increased levels of Rac at the PM in Abca1−/− Abcg1−/− macrophages. Recently we showed that the combined deficiency of ABCA1 and ABCG1 in hematopoietic stem cells leads to an increase in Ras protein in the PM in vivo promoting hyper-proliferation in response to growth factors. In addition, Abca1−/− Abcg1−/− macrophages show increased Nox2 assembly in the PM and in phagolysosomes as macrophages ingest apoptotic cells. Because the assembly of Nox2 complexes is dependent on the membrane recruitment of Rac1, increased Rac1 activation may also be involved in increased Nox-2 assembly in Abca1−/− Abcg1−/− macrophages. Thus, HDL acting in conjunction with ABCA1/G1 may serve to limit formation of cholesterol-rich domains on the inner leaflet of the PM of cholesterol-loaded macrophages, and thus modulate signaling via small GTPases such as Ras and Rac1, affecting myeloid cell proliferation, as well as cell motility and reactive oxygen species formation during effectorcytosis in macrophages.

The progression of atherosclerotic lesions is marked by an inability of macrophage foam cells to leave the artery. By using a well-described peritoneal cavity emigration model, we showed that macrophages from Abca1−/− Abcg1−/− mice have a substantial defect in their migration behavior in vivo. In contrast to the present findings, studies in resident macrophages or in blood monocytes show that cholesterol removal via the ABCA1/apoA1 pathway can inhibit migration. Thus, ABC transporters and HDL may act to inhibit monocytosis and ABCA1/apoAI pathway can inhibit migration. Thus, ABC transporters and HDL may act to inhibit monocytosis and ABCA1/apoAI pathway can inhibit migration.

Disclosures

Sources of Funding

This work was supported by NIH grants HL54591 (to A.R.T.), AG20255 (to K.J.M.), and DK27083 (to F.R.M.); and American Heart Association Predoctoral Fellowship 10PRE3020048 (to M. Wang) and American Heart Association Postdoctoral Fellowship 09POST2110109 (to M. Westerterp). T.A.P. was supported by the Fonds zur Förderung der Wissenschaftlichen Forschung (FWF)–“Erwin Schrödinger” Stipend, J2791-B13.

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Tall AR. ABCA1 and ABCG1 promote cholesterol movement from the inner to the outer leaflet of plasma membranes. In Recent studies in mice have shown that increased HDL may promote emigration of monocyte/macrophages from atherosclerotic plaques. A major property of HDL is its ability to mediate cholesterol efflux from macrophage foam cells in part by promoting cholesterol efflux from macrophage foam cells via the ATP-binding cassette transporters ABCA1 and ABCG1.

Novelty and Significance

What Is Known?

- High-density lipoprotein (HDL) exerts antiatherogenic effects in part by promoting cholesterol efflux from macrophage foam cells via the ATP-binding cassette transporters ABCA1 and ABCG1.
- Increasing HDL-cholesterol concentrations promotes regression of atherosclerosis in mouse models, and this is associated with emigration of macrophages from lesions.

What New Information Does This Article Contribute?

- Abca1−/− Abcg1−/− macrophages have severely impaired chemotaxis attributable to plasma membrane cholesterol accumulation and excessive Rac-1 activation.
- HDL can improve impaired chemotaxis in cholesterol loaded wild-type macrophages, but not in Abca1−/− Abcg1−/− macrophages.
- ABCA1 and ABCG1 promote cholesterol movement from the inner to the outer leaflet of plasma membranes, and thus maintain correct localization and activity of Rac-1.

Therapeutic approaches to raising HDL-cholesterol concentrations are at the forefront of atherosclerosis clinical research. Recent studies in mice have shown that increased HDL may promote emigration of monocyte/macrophages from atherosclerotic plaques. A major property of HDL is its ability to mediate cholesterol efflux from macrophage foam cells in part by promoting cholesterol efflux from macrophage foam cells via the ATP-binding cassette transporters ABCA1 and ABCG1. We evaluated the hypothesis that macrophage chemotaxis might be promoted by ABCA1/G1 and HDL. We showed that HDL could reverse severely impaired chemotaxis of cholesterol-loaded wild-type macrophages, but not of macrophages lacking these ABC transporters. In further studies, we used a novel biophysical technique to demonstrate that ABCA1/G1 promote cholesterol movement from the inner to the outer leaflet of the plasma membrane. In the absence of transporters, sterol accumulation in the inner membrane leads to increased Rac-1 activity and thus impaired chemotaxis. The modulation of small GTPases that are membrane tethered appears to be a fundamental cellular effect of HDL and the ABC transporters that influences a variety of cellular processes including cell motility and proliferative responses. Thus, this study elucidates basic cellular mechanisms that are directly relevant to mechanisms by which increasing plasma HDL concentrations may produce regression of atherosclerotic plaques.
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Circ Res. 2011;108:194-200; originally published online December 9, 2010; doi: 10.1161/CIRCRESAHA.110.228619

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Supplemental Material

Supplemental Methods

Materials. HDL (density 1.063-1.21 g/ml) was isolated by preparative ultracentrifugation from normolipidemic human plasma and stored in PBS. Human recombinant C5α, filipin, 2,4,6-trinitrobenzenesulphonic acid, T0901317 and the Rac inhibitor NSC23766 were purchased from Sigma. TRITC-labeled phalloidin was purchased from Lonza. Fluorescence beads were obtained from Polysciences. Rac1-GTP and RhoA-GTP luminometric or colorimetric ELISA kits were purchased from Cytoskeleton Inc. The antibody rabbit anti-mouse Rac1/2/3 was from Cell Signaling Technology, anti-rabbit β-actin was purchased from Sigma and the APC-F4/80 was purchased from e-Bioscience. Cholestatrienol was kindly provided by Dr. J. David Warren (Weill Cornell Medical College).

Macrophage harvest and treatment. Peritoneal macrophage cells were harvested from WT, and Abca1−/−Abcg1−/− mice 3 days after receiving an i.p injection of thioglycollate and plated in DMEM media containing 10% FBS as previously described.1 Adherent cells consisting of macrophages were used for experiment. Where indicated, macrophages were pre-incubated with 10nmol/L Rac1 inhibitor NSC23766 for 30min or 5mmol/L chol-MβCD in 20 mmol/L HEPES-buffered DMEM for 30 min or 100µg/ml HDL before subjection to migration as described in figure legends. For plasma membrane bilayer sterol distribution experiment cells were treated over night where indicated with 50µg/ml AcLDL and 3µmol/L T0901317.

Transwell migration assay. Freshly isolated macrophages from WT and Abca1−/−Abcg1−/− were seeded for 2 hours in regular growth media as described above. Subsequently cells were washed and adhered macrophages were treated with trypsin (Sigma) and reseeded at a density of 0.5Mio/well on top of 5-µm polycarbonate filter inserts in Transwell chamber plates (Costar) and incubated for 2h.Where indicated cells were treated with 5mmol/L cholesterol-methyl-β-cyclodextrin in 20 mmol/L HEPES-buffered DMEM for 30 min, and then washed once with DMEM. Migration capacity was tested by placing the filter inserts with cells in wells containing 700µl of 10 nmol/L C5a diluted in DMEM. Migration was allowed to proceed for 2 h at 37° C in a CO2 incubator. After this incubation period, the filters were fixed with 4% paraformaldehyde, and cells that had not migrated were removed from the upper surface of the filter by scraping using Q-Tips. The filters were then stained with 1µl/ml Hoechst (Sigma), mounted onto coverslip dishes, and observed via confocal microscopy (Axioskop 2 FS MOT upright confocal microscope (Zeiss)). The numbers of cells that had migrated across the filters were determined by counting the number of cells in at least 6 random fields of images acquired with a 10x objective at the bottom of each filter. During cholesterol deoading treatment, cells were incubated with10 mM MβCD for 10 minutes to lower cholesterol levels.

In vivo migration. For in vivo migration experiments, fluorescence labeled beads (250µl of a 40µl beads in 1ml in sterile phosphate buffer saline dilution) were injected into the peritoneal cavity of 72h post-thioglycollate stimulated WT or Abca1−/−Abcg1−/− mice. One hour later cells were isolated from the peritoneal cavity and stained for the macrophage marker F4/80. Using FACS, macrophages staining positively for TRITC (green) or PE (red) were identified with APC-labeled F4/80 and sorted (BD FACSAria Cell Sorter). Three hours later macrophages positive for bead label and F4/80 were re-injected into mice bearing ongoing inflammation triggered by thioglycollate injection at 72h before. Remaining numbers of labeled macrophages were determined by FACS 12 hours later as % of total macrophages.
F-actin and filipin staining. Freshly isolated macrophages were seeded on cover slips for 12h. Subsequently cells were fixed with 3.3% paraformaldehyde in the presence of 0.25 mg/mL saponin and 1U/mL TRITC-phalloidin in phosphate buffered saline for 15 minutes at room temperature. The coverslips were mounted and visualized with a Axioskop 2 FS MOT upright confocal microscope (Zeiss). Images were obtained by implementing z-scanning and analyzed using the ImageJ software. At least three separate fields from triplicate wells for each genotype were analyzed per experiment. To quantify changes in free cholesterol at the plasma membrane, cells were fixed with 4% paraformaldehyde for 15 minutes and then incubated with 50 µg/mL filipin for 1.5h at room temperature. Wide-field images were obtained and the MetaMorph image analysis software was used to analyze the images. Images were background corrected followed by the measurement of the average fluorescence intensity along the periphery indicating the plasma membrane of the cell for more than 30 cells per genotype.

Rac1- and RhoA-GTP ELISA. Peritoneal macrophages were seeded in 12-well plates overnight in regular growth media. Followed by a starvation period of 24h using DMEM containing 0.2% fatty acid free BSA (Sigma). Cells were then lysed in lysis buffer and Rac or RhoA-GTP levels were measured following manufactures instructions (Cytoskeleton Inc.).

Plasma membrane fractionation. Thioglycollate elicited cells were seeded overnight in regular growth media. This was followed by a 48h starvation period in DMEM media containing 0.2% fatty acid free BSA. Subsequently cells were lysed using a 20mmol/L HEPES buffer at pH 5.5 and subjected to homogenization using a Dounce homogenizer (20 strokes). Nuclei were removed by spinning lysate for 10min at 14,000 rpm. To collect the plasma membrane fraction supernatant was centrifuged at 70,000 rpm for 20min at 4°C in an ultra-centrifuge.

Western blot analysis. Plasma membrane fractions were electrophoresed on 4-20% gradient SDS-PAGE gels and transferred to 0.22-µm nitrocellulose membranes. The membrane was blocked in Tris-buffered saline, 0.1% Tween20 (TBST) containing 5%(w/v) nonfat milk at room temperature (RT) for 1h and then incubated with the primary antibody in TBST with 5% fatBSA at room temperature over night, followed by incubation with the appropriate secondary antibody coupled to horseradish peroxidase. Proteins were detected by ECL chemiluminescence (Pierce).

Sterol transbilayer distribution. The distribution of the fluorescence cholesterol analogue cholestatrienol between the inner and the outer leaflet of the plasma membrane bilayer was determined as described previously. In brief, cells were trace-labeled with CTL-MβCD complexes (0.5mmol/L) for 1min at 37°C washed, and then incubated for 10 min at 37°C in order to reach a steady-state distribution of CTL. Images in identical regions were acquired before and within 30 s after on stage addition of the plasma membrane impermeable quencher TNBS. Fluorescence microscopy and digital image acquisition were carried out using a Leica DMRB microscope (Leica) with a CCD camera optimized for CTL imaging. All images were acquired using a 63x, 1.4 NA oil immersion objective. For fluorescence intensity measurements images were first background-corrected and corrected for photobleaching as described. Boxes were drawn in peripheral regions of the cells to measure the plasma membrane associated CTL fluorescence. At least 30 cells per conditions were analyzed in each experiment.

Statistical analysis. Data are given as means ± S.E.M. Comparison of mean values between groups was evaluated by two-tailed parametric student’s t test or by one-way analysis of variance (ANOVA, 4-group comparisons) with a Bonferroni multiple comparison post-test (GraphPad software, San Diego, CA). Differences were considered significant at a p<0.05.
Supplemental References


Supplemental Figure I. Abca1−/−Abcg1−/− macrophages show impaired transwell migration towards various chemoattractant. Thio-elicited macrophages from WT or Abca1−/−Abcg1−/− mice were seeded on Transwell filters and the migration capacity was tested by stimulation with 10nM C5a for 2h or 50nM CCL21 or 10nM MCP-1 for 5h. Migrated macrophages were quantified after fixation and DAPI staining. Results were normalized to the number of untreated WT macrophages that migrated for each condition separately. Data shown are mean ± SEM of experiments performed in triplicates, * p<0.05 vs. WT +MCP-1.
Abca1⁻/⁻ and Abcg1⁻/⁻ macrophages do not show impaired transwell migration. Migratory capacity of WT, Abca1⁻/⁻ or Abcg1⁻/⁻ macrophages towards C5a was tested as described in Suppl. Fig. 1. Results were normalized to the number of untreated WT macrophages that migrated. Data shown are mean ± SEM of experiment performed in triplicates.
Supplemental Figure III. MβCD but not HDL treatment reversed the migratory defect of Abca1<sup>-/-</sup> Abcg1<sup>-/-</sup> macrophages. Thio-elicited macrophages from WT or Abca1<sup>-/-</sup> Abcg1<sup>-/-</sup> macrophages were seeded on transwell filters and the migration capacity was tested using C5a after indicated treatments. The results shown are mean ± SEM of experiment performed in triplicates. **: P<0.01 compared with Abca1<sup>-/-</sup> Abcg1<sup>-/-</sup> macrophages with C5a but no other treatment. #: P<0.05 compared with Abca1<sup>-/-</sup> Abcg1<sup>-/-</sup> macrophages MβCD pretreatment and C5a.
Supplemental Figure IV. *Abca1*<sup>−/−</sup>*Abcg1*<sup>−/−</sup> macrophages show increased plasma membrane ruffling and cell spreading. Thio-elicited macrophages from WT and *Abca1*<sup>−/−</sup>*Abcg1*<sup>−/−</sup> mice were seeded for 12h before fixation and staining for F-actin using TRITC-phalloidin. 3D-projected confocal images show a marked increase in cell membrane ruffles in *Abca1*<sup>−/−</sup>*Abcg1*<sup>−/−</sup> macrophages indicated by white arrows as well as an increase in lamellipodia formation surrounding the cells as indicated by arrow heads.
Supplemental Figure V. *Abca1*<sup>-/-</sup> and *Abcg1*<sup>-/-</sup> macrophages do not have altered Rac1 activation. Thioglycollate-elicited macrophages from WT, *Abca1*<sup>-/-</sup> (A) or *Abcg1*<sup>-/-</sup> (B) mice were serum depleted for 24h before cell lysates were analyzed for Rac1-GTP levels using a commercial available ELISA kit. Data shown are mean ± SEM of experiment performed in triplicates.
Supplemental Figure VI. *Abca1*<sup>−/−</sup>*Abcg1*<sup>−/−</sup> macrophages exhibit similar levels of RhoA-GTP than WT. Thioglycollate-elicited macrophages from WT and *Abca1*<sup>−/−</sup>*Abcg1*<sup>−/−</sup> mice were seeded over night and subsequently starved for 24h before cell lysates were analyzed for Rac1-GTP levels using a commercial available ELISA kit. The results shown are mean ± SEM of experiments performed in triplicates.
Supplemental Figure VII. *WT* macrophages treated with either AcLDL or LXR agonist alone do not show a significant increase in outer leaflet cholesterol accumulation. Transbilayer plasma membrane cholesterol distribution was measured in macrophages from WT mice treated either with AcLDL or LXR agonist alone or in combination as described in Fig. 7 of the manuscript. A total of 50-60 cells for each treatment condition were analyzed. Data are shown as mean ± SEM. *** p<0.001 vs. basal.
Supplemental Figure VIII. Sterol mobility in the PM is unaffected in \textit{Abca1}^{−/−} \textit{and Abcg1}^{−/−} macrophages. Transbilayer plasma membrane cholesterol distribution was measured in macrophages from \textit{Abca1}^{−/−} \textit{and Abcg1}^{−/−} mice as described in Fig. 7. A total of 30–50 cells for each treatment condition were analyzed. Data are shown as mean ± SEM. * p<0.05 vs. basal, *** p<0.001 vs. basal.