Rab8 Regulates ABCA1 Cell Surface Expression and Facilitates Cholesterol Efflux in Primary Human Macrophages

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Objective—ATP-binding cassette transporter A1 (ABCA1) is thought to lipitate apolipoprotein A-I (apoA-I) at the plasma membrane, with endosomal cholesterol contributing as substrate. The mechanisms of ABCA1 surface delivery are not well understood. We have shown that Rab8 regulates endosomal cholesterol removal to apoA-I in human fibroblasts. Here, we investigated whether Rab8 plays a role in ABCA1 plasma membrane expression and cholesterol removal in primary human macrophages.

Methods and Results—We found that Rab8 was abundantly expressed in human atherosclerotic lesional macrophages and upregulated on lipid loading of macrophages in vitro. Adenoviral overexpression of Rab8 increased ABCA1 protein levels and reduced cholesterol deposition in macrophage foam cells incubated with apoA-I. Depletion of Rab8 decreased the fraction of ABCA1 at the plasma membrane and inhibited the efflux of lipoprotein-derived endosomal cholesterol to apoA-I. In Rab8-depleted cells, ABCA1-GFP localized in β1 integrin and transferrin receptor containing recycling organelles.

Conclusion—Rab8 reduces foam cell formation by facilitating ABCA1 surface expression and stimulating endosomal cholesterol efflux to apoA-I in primary human macrophages. (Arterioscler Thromb Vasc Biol. 2009;29:883-888.)

Key Words: Rab GTPase ■ foam cell ■ apolipoprotein A-I ■ ATP-binding cassette transporter

Due to the formation of an atherosclerotic plaque, monocyte-derived macrophages invade the arterial wall where they scavenge lipoprotein particles retained in the intima. This results in excessive cholesterol deposition in the macrophages that transform into lipid-laden foam cells. The internalized cholesterol in turn elicits adaptive responses, such as activation of cholesterol storage and removal systems.1 Efflux of cholesterol from macrophage foam cells to apolipoprotein A-I (apoA-I), the major apolipoprotein of high-density lipoprotein (HDL), is thought to represent a critical step in macrophage reverse cholesterol transport2 and one of the key mechanisms in the antiatherogenic action of HDL.3 Lipid-poor apoA-I interacts with the ATP-binding cassette (ABC) transporter A1 (ABCA1) on the cell surface to promote net cholesterol efflux to apoA-I.4 In macrophages lacking ABCA1 function, cholesterol and phospholipid efflux to apoA-I is severely attenuated, and massive amounts of cholesterol are stored as fatty acid esters in lipid droplets.5,6

In comparison to the plasma membrane events in cholesterol efflux, the intracellular mechanisms that govern ABCA1 trafficking to the cell surface and cholesterol delivery for efflux are poorly understood. Endosomal compartments are apparently involved. There is evidence that acetylated LDL (acLDL)-derived cholesterol deposited in late endosomes/lysosomes is a significant source of cholesterol for ABCA1-mediated cholesterol efflux.7 Deletion of the PEST sequence of ABCA1 impaired its transport to late endosomes and consequently reduced the efflux of acLDL-derived cholesterol to apoA-I.8 Moreover, we found that in human fibroblasts the delivery of cholesterol from late endosomal compartments to apoA-I was stimulated on overexpression of the small GTPase Rab8.9

Rab8 belongs to the large family of Rab GTPases that regulate various stages of vesicular transport or membrane-cytoskeleton interactions.10 So far, no Rab have been reported as regulators of macrophage cholesterol trafficking. In earlier studies, Rab8 has been implicated in cytoskeletal organization and membrane trafficking to the cell surface,11–13 including specialized plasma membrane domains.14–17 We therefore considered that Rab8 might also be involved in regulating the delivery of cholesterol via ABCA1 to apoA-I in human monocyte-derived macrophages. In this work, we studied the expression of Rab8 in macrophages and analyzed the effects of altered Rab8 expression on ABCA1 localization and macrophage cholesterol processing.

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Methods

A detailed Materials and Methods section is posted as an online supplement (available at http://atvb.ahajournals.org).

Isolation of Primary Human Macrophages

Human monocytes from healthy control subjects were isolated fromuffy coat cells (Finnish Red Cross Blood Transfusion Service) as described. Monocytes were allowed to adhere to the flask for 60 minutes in RPMI medium, washed once with PBS, and differentiated into macrophages for 7 days in macrophage serum free medium (M-SFM) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 10 ng/mL GM-CSF.

RNA Interference and Cholesterol Measurements in Primary Human Macrophages

Control and Rab8A-specific small interfering RNAs (siRNA) have been described. SiRNA targeting human Rab8b was from Ambion (predesigned Silencer oligo 24779). On day 0, differentiated macrophages were collected by trypsinization and electroporated with control, Rab8A-, or Rab8b-specific siRNA using Human macrophage nucleofection kit (Amaxa). After electroporation, cells were incubated in M-SFM +10% FBS for 18 hours, and on day 1 the medium was changed to M-SFM only. On day 2, the cells were loaded with 50 μg/mL acLDL for 24 hours and incubated with 10 μg/mL apoA-I for 18 hours. Cells were harvested in 1% Nonidet P-40 in PBS, and protein concentration was determined using the DC Protein Assay (Bio-Rad). Cholesterol amounts were measured using the Amplex Red Cholesterol Assay Kit (Invitrogen). For measurement of total [3H]-cholesterol, equal amounts of material (corrected for protein amounts) was counted by liquid scintillation counting.

Surface Biotinylation

Differentiated macrophages were electroporated as above. On day 2, the cells were incubated with 50 μg/mL acLDL and 10 μg/mL apoA-I for 24 hours. Surface biotinylation was carried out as described except that EZ-Link Sulfo-NHS-SS-Biotin (Pierce) was used. One-tenth of the volume was removed to represent the “total” sample and 9/10 was precipitated with Streptavidin-agarose (Pierce) overnight at 4°C, followed by washes (once with lysis buffer, 3 times with 1% NP40 in PBS, twice in 0.1% NP40, 0.5 mol/L NaCl in PBS, twice with 50 mmol/L Tris–HCl, pH 7.5), and Western blotting. The fraction of ABCA1 on the cell surface was calculated by comparing the total versus biotinylated ABCA1 for each donor. To allow for comparison between macrophages from individual donors, the fraction of ABCA1 on the cell surface in control siRNA treated cells was set as 1.0.

Results

Rab8 Is Abundantly Expressed in Human Atherosclerotic Lesional Macrophages

To assess Rab8 expression in a physiological setting of foam cell formation, we stained sections of atheromatous human coronary arteries with anti-Rab8 antibodies. Marked Rab8 immunoreactivity was observed in the arterial intima, including cells with macrophage-like morphology that infiltrated the atheroma plaque. Prominent Rab8 immunoreactivity was observed in the cytoplasm, in particular in cells surrounding the necrotic lipid core (Figure 1A and 1C). Costaining with anti-CD68 antibody demonstrated significant overlap with anti-Rab8 immunostaining (Figure 1B), indicating that the highly Rab8 positive cells were indeed macrophages.

Lipid-Loaded Macrophages Target Rab8 and Cholesterol to the Leading Edge

To elucidate the role of Rab8 in macrophages, we studied its expression levels and localization. Human peripheral blood monocytes were differentiated into macrophages in vitro and loaded with acLDL to induce foam cell formation. When the expression levels of Rab8 were analyzed by Western blotting, we found Rab8 to be upregulated by approximately 2-fold on acLDL loading and to be downregulated after lipid unloading with apoA-I (Figure 2A). The monocyte-derived macrophages underwent marked morphological changes on challenging with acLDL and apoA-I. The cells extended cholesterol-rich protrusions as identified by filipin staining (Figure 2B and 2D) and elongated (Figure 2C). This phenotype could also be achieved by incubating macrophages with acLDL or apoA-I alone (Figure 2C). Indeed, modified LDL is known to stimulate macrophage migration. Motile cells are characteristically asymmetrical, with a retractile tail and a leading edge that harbors...
lamellipodial and filopodial extensions and cholesterol-rich membrane microdomains. Rab8 has been localized to the perinuclear Golgi and recycling endosome compartments as well as to the plasma membrane. We found that in macrophages, acLDL and apoA-I induced the formation of lamellipodia and membrane ruffles that harbored considerable amounts of Rab8 as detected by immunostaining of the endogenous protein (Figure 2D).

Rab8 Overexpression Increases ABCA1 Levels and Reduces Cholesterol Deposition in Foam Cells

Rab8 overexpression is known to induce the formation of cell protrusions. Rab8 has been localized to the perinuclear Golgi and recycling endosome compartments as well as to the plasma membrane. We found that in macrophages, acLDL and apoA-I induced the formation of lamellipodia and membrane ruffles that harbored considerable amounts of Rab8 as detected by immunostaining of the endogenous protein (Figure 2D).

Figure 2. Upregulation and targeting of Rab8 to the leading edge of macrophage foam cells. Anti-Rab8 Western blot (A) and filipin staining of macrophages (B). C, Quantification of macrophage elongation (n=50, ***P<0.001). D, Filipin and anti-Rab8 antibody staining of macrophages. Bars, 20 μm (B) and 10 μm (D).

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Rab8 Depletion Aggravates Foam Cell Cholesterol Deposition and Inhibits Cholesterol Efflux to ApoA-I

Next, we depleted endogenous Rab8 from human primary macrophages using siRNAs. Rab8 antibodies visualized a doublet of bands at ~25kDa in Western blots (Figure 4A), with the upper band corresponding to Rab8A and the lower to the Rab8b isoform. Specific siRNAs for separately knocking down each isoform were generated and introduced into differentiated macrophages for 2 days. This typically resulted in ~70% depletion of the target protein isoform with no apparent change in the level of the other isoform (Figure 4A).

Rab8A siRNA or control siRNA-treated cells were loaded with acLDL overnight. In control siRNA-treated cells, this increased the total cellular cholesterol levels about 3-fold and the fraction of cholesteryl esters from ~3.5% in the basal situation to ~45% after acLDL loading. Rab8A-depleted cells were cholesterol loaded on acLDL incubation to a similar extent as the control cells (Figure 4B). Control and Rab8A siRNA-treated cells were then incubated in the presence of apoA-I overnight to induce net cholesterol efflux. In control siRNA-treated cells, a significant reduction in the total cholesterol level was achieved (Figure 4B). In contrast, there was essentially no reduction in the sterol levels of Rab8A siRNA-treated cells after the apoA-I incubation (Figure 4B), and a major fraction of the sterol remained as fatty acid esters (~48% and ~40% in Rab8A and control siRNA treated cells, respectively). In contrast, depletion of the Rab8b isoform did not affect cholesterol levels on acLDL loading or incubation with apoA-I and acLDL, except that the phenotype was more exaggerated, with multiple extensions pointing to several directions (data not shown). Moreover, when the cellular ABCA1 amounts were analyzed, we observed a striking increase in ABCA1 levels in the Rab8-virus infected cells as compared to GFP-virus infected cells (Figure 3B). To determine whether this was attributable to increased ABCA1 gene transcription we performed quantitative real-time PCR on control or Rab8 infected macrophages. The ABCA1 mRNA levels were not altered, suggesting that the half-life of the ABCA1 protein is increased in Rab8 overexpressing cells (Figure 3C).

To study the effect of Rab8 overexpression on ABCA1-dependent cholesterol efflux, we measured the efflux of [3H]-cholesterol from infected macrophages to apoA-I. Rab8 overexpression increased [3H]-cholesterol efflux to apoA-I by ~20% as compared to control (Figure 3D). To assess the effect of Rab8 overexpression on foam cell cholesterol processing, the adenovirally infected cells were loaded overnight with acLDL in the presence of apo-A-I. The amount of cholesterol deposited in the cells was then determined. In control adenovirus infected cells, the total cholesterol levels increased about 1.7-fold during this treatment. We found that the Rab8-overexpressing cells accumulated slightly less cholesterol than the control infected cells despite the continuous cholesterol challenge (Figure 3E). In this setting, Rab8 overexpression primarily affected the cholesteryl ester pool. The lipid challenge increased the cholesteryl ester stores of GFP-expressing cells about 8-fold, whereas in Rab8 overexpressing cells the cholesteryl ester stores increased only about 5-fold, i.e., there was a ~40% reduction in cholesteryl esters (Figure 3F).
apoA-I efflux, and the combined effect of Rab8A and Rab8b depletion was not more pronounced than that obtained with Rab8A depletion alone (data not shown). Notably, also in other systems, differential roles for the Rab8A and Rab8b isoforms have been reported. Consequently, we next focused on the effects of Rab8A depletion.

To investigate whether the pool of cholesterol endocytosed via scavenging of modified lipoproteins represents a pool of cholesterol that is affected by Rab8 siRNAs, we loaded control or Rab8 siRNA treated cells with [3H]-cholesteryl oleate labeled acLDL, followed by cholesterol efflux to apoA-I as described above. This radiolabeled cholesteryl ester pool is hydrolyzed by lysosomal acid lipase followed by transfer of free [3H]-cholesterol to extracellular acceptors, and can therefore be used to trace cholesterol efflux from late endosomes. We found that [3H]-cholesteryl oleate loading of Rab8A siRNA-treated cells was comparable to that of control siRNA-treated cells but that the efflux of the [3H]-cholesterol from Rab8-depleted cells was reduced as compared to the control cells (Figure 4C and 4D). This suggests that defective mobilization of the late endosomal cholesterol pool to apoA-I significantly contributes to the cholesterol deposition phenotype observed in Rab8-depleted macrophages.

**Rab8 Depletion Reduces ABCA1 at the Plasma Membrane and Affects its Intracellular Routing**

ABCA1-mediated lipidation of apoA-I is thought to take place at the plasma membrane. Because Rab8 can regulate protein delivery to the cell surface, we considered that Rab8 depletion might reduce cholesterol efflux to apoA-I by interfering with the cell surface exposure of ABCA1. To determine the amounts of ABCA1 at the plasma membrane, we performed surface biotinylation of Rab8 knockdown and control macrophages followed by anti-ABCA1 Western blotting. This revealed a marked reduction in the fraction of ABCA1 protein at the plasma membrane in Rab8 depleted cells compared to control cells (Figure 5A and 5B).
cholesterol deposition when exposed to modified LDL. Because of these findings and the discovery that Rab8 was robustly expressed in macrophages of human atherosclerotic plaques, Rab8 is likely to represent an important player in the pathophysiology of atherosclerosis.

In Tangier disease cells as well as in macrophage foam cells, most of the cholesterol accumulates as cholesteryl esters in lipid droplets, which helps to prevent the accumulation of excess unesterified cholesterol.26 Therefore, it is worth noting that we found Rab8 overexpression to effectively reduce the cholesteryl ester stores in foam cells. Because Rab8 helps to shunt endocytosed cholesterol for efflux, less cholesterol might be available for storage in lipid droplets as cholesteryl esters. Rab8 overexpression might also more directly facilitate the mobilization of lipid droplet cholesterol, eg, via the Golgi apparatus. These scenarios are not mutually exclusive, ie, both the endosomal and lipid droplet cholesterol pools may serve as sources for ABCA1-mediated, Rab8-stimulated cholesterol efflux.

An interesting question arising from the present study is how Rab8 controls the cell surface accessibility of ABCA1 and cholesterol removal from endosomal circuits to apoA-I. One possibility is that the primary effect of Rab8 is the mobilization of endosomal cholesterol, and this affects ABCA1 levels and localization by regulatory mechanisms, such as liver X receptor activation via the generation of oxysterols. However, Rab8 overexpression was found to markedly increase the amount of ABCA1 protein without a corresponding upregulation of the mRNA, rather arguing for a more direct posttranslational role for Rab8. It seems likely that Rab8 is involved in the transport/recycling of ABCA1 to the cell surface. Supporting this view, Rab8 siRNA decreased the proportion of ABCA1 at the cell surface and caused the retention of ABCA1-GFP in β1 integrin containing intracellular tubules and TIR-positive recycling compartment.

ABCA1-GFP has been localized to endosomes and the plasma membrane25 but how ABCA1 cycles between them is not clear. The colocalization of ABCA1-GFP with β1 integrin in control and Rab8-depleted cells suggests that ABCA1 recycles in part along the same route as β1 integrin. Rab8 depletion also caused some ABCA1-GFP to redistribute to a perinuclear TIR-positive recycling compartment. Because ABCA1-GFP does not normally reside in TIR-positive vesicles,26 this reinforces the idea of altered ABCA1 trafficking on Rab8 depletion. Of note, the transport routes of β1 integrin and TIR are related as the proteins partially recycle via the same compartments and are in part regulated by the same GTPases.27 Moreover, syntaxin-13, which has been shown to interact with ABCA1,28 also regulates TIR recycling.29

In several cell types, Rab8 regulates cell shape, particularly the formation of specialized cell surface extensions that rely on coordinated cytoskeletal organization and membrane transport.12,13,15,16 We found that on challenging with modified LDL and apoA-I, macrophages elongated and developed a migratory phenotype with a leading edge and a tracking tail. Also, cell motility increased (our unpublished observations). Moreover, Rab8, ABCA1, and β1 integrin were concentrated at the leading edge of cells. Together with the data on cholesterol efflux, our results suggest that membrane protrusion...
sions at the leading edge may function as preferential plasma membrane domains for cholesterol efflux to apoA-I. This agrees with earlier studies in which ABCA1 was found to interact with scaffolding molecules linking to the cortical actin cytoskeleton, or the small GTPase Cdc42 that regulates the formation of filopodia. Considering the involvement of Rab8 in cytoskeletal regulation, it is also conceivable that Rab8 contributes to the stabilization of ABCA1 at the plasma membrane.

In summary, our results indicate that Rab8 is one of the intracellular regulators that promote ABCA1 plasma membrane localization and govern the delivery of cholesterol to apoA-I in macrophages. Interestingly, Rab8 has also been reported to regulate the exocytic delivery of MT1-matrix metalloproteinase during cell invasion, a process that is critical for the infiltration of macrophages into atherosclerotic lesions. Given the dual role for Rab8 in matrix metalloproteinase release and ABCA1-mediated cholesterol efflux by tissue-infiltrating cells, it seems plausible that these processes function in a coordinated fashion to facilitate cholesterol scavenging and efflux in the arterial wall.

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Disclosures
None.

References
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Supplemental Material

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Materials and Methods

Materials

Cell culture media, defatted bovine serum albumin (BSA), protease inhibitors, anti-FLAG antibodies and filipin were from Sigma-Aldrich. Lipid-free human apoA-I was provided by Peter Lerch (Swiss Red Cross, Bern, Switzerland). Alexa conjugated secondary antibodies was from Invitrogen. Anti-Rab8 monoclonal antibodies were from BD Biosciences. Anti-Rab8 rabbit polyclonal antibodies have been described \(^1\). Anti-ABCA1 monoclonal antibodies were from Abcam. Anti-Transferrin receptor antibodies were from Zymed. β1 integrin antibodies were provided by Ismo Virtanen \(^2\). ABCA1-GFP was from Jonathan D. Smith \(^3\), and ABCA1-FLAG from Alan R. Tall \(^4\). Macrophage serum-free medium (M-SFM) was from Gibco. Recombinant Human Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) was from Invitrogen. LDL was provided by Matti Jauhiainen (National Public Health Institute, Helsinki, Finland). AcLDL was prepared and labeled with \([1\alpha,2\gamma(n)\text{H}]\)-cholesteryl oleate (Amersham) as described \(^5\).

Immunohistochemistry

Formalin-fixed, paraffin-embedded human coronary artery sections were obtained as described \(^6\). Anti-Rab8 antibody was detected using the avidin-biotin system (Vectastain ABC Elite rabbit kit, Vector Laboratories) and 3,3’-diaminobenzidine (Sigma-Aldrich).
Sections were counterstained with Mayer's hematoxylin. Specificity of the affinity purified Rab8A antibody was confirmed by blocking with purified recombinant NusA-Rab8A protein before processing as above. For double staining, sections were stained with rabbit anti-Rab8 and anti-CD68 (Dako, clone PG-M1). Antibodies were visualized using goat-anti rabbit IgG fluorescein isothiocyanate and goat-anti mouse Alexa594. Sections were counterstained with 4’,6-diamidino-2-phenylindole (Sigma-Aldrich). Samples were photographed with a digital camera (Spot RT color operated with Spot advanced software, Version 4.6, Diagnostic Instruments) attached to a Nikon Eclipse E600 microscope.

**Immunocytochemistry and microscopy**

For staining with anti-Rab8, β1 integrin or transferrin receptor antibodies, paraformaldehyde fixed cells were permeabilized with 0.1% Triton X-100 and blocked with 10% FBS in PBS. For visualization of free cholesterol, cells were stained with 0.05% filipin in blocking solution for 30 min at 37°C. Photomicrographs of filipin stained cells were taken with an Olympus AX70 microscope equipped with an Olympus DP71 CCD camera. All others (except Fig. 1) were taken with a Leica SP2 confocal microscope.

**Cell culture and transfection of HeLa cells**

HeLa cells were obtained from ATCC and grown in D-MEM supplemented with 100U/mL penicillin, 100µg/mL streptomycin and 10% FBS. SiRNA was introduced to
cells by transfection with HiPerFect (Qiagen) and overexpression was done with Lipofectamine according to the manufacturer’s instructions.

**Western blotting**

Cells were lysed in 1% Nonidet P-40 in PBS+CLAP. Equal amounts of cellular protein (15 µg per sample) were subjected to SDS-PAGE and Western blotting as described \(^8\).

**Adenovirus infection**

Human Rab8 cDNA was cloned into the pAdEasy-1 vector and recombinant Rab8A adenovirus was generated in HEK293 cells using the AdEasy™ Adenoviral Vector System (Stratagene). Recombinant viruses were expanded, purified using the Vivapure AdenoPACK 100 kit (Generon), and titrated on HEK293 cells with the Adeno-XTM rapid titer kit (Clontech). Macrophages were infected with control virus expressing GFP or Rab8A adenovirus at a M.O.I. of 600, which achieved a transduction of > 60% of cells. Three days post-infection, cells were processed for Western blotting or quantitative real-time PCR as described \(^9\). For cholesterol measurements, cells were incubated at two days post infection with 50µg/ml acLDL and 10 µg/ml apoA-I for 24h. Cholesterol and cholesteryl esters were determined as above. For \(^{3}H\)-cholesterol efflux measurements, macrophages were infected as above. On day 2 post-infection, cells were labeled with 1 µCi /ml 1,2-[\(^{3}H\)]-cholesterol (Amersham) for 18 h, and then incubated in the presence of 10 µg/ml ApoA-I for 8h. The \(^{3}H\)-radioactivity in the medium and cells was determined by liquid scintillation counting. The efflux % was calculated as (DPM\(_{\text{medium}}\)/ (DPM\(_{\text{cells}}\) + DPM\(_{\text{medium}}\)) x 100.
Statistical analysis

Results are expressed as average±S.E.M. Statistical significance was determined using Student’s t test; p < 0.05 was considered statistically significant.

References


**Extended Figure Legends**

**Figure 1. Rab8 protein in macrophage foam cells in vivo.** (A) Immunohistochemical staining of a human coronary artery section with anti-Rab8 antibodies; the boxed areas are shown below with higher magnification. Arrows indicate anti-Rab8 positive cells. (B) Double staining of a human coronary artery section with anti-CD68 and polyclonal anti-Rab8 antibodies followed by Alexa 594- and FITC-conjugated secondary antibodies, respectively. Nuclei were stained with DAPI. Co-localization of CD68 (red) and Rab8 (green) is visualized as yellow; the boxed area is shown with higher magnification. (C)
Specificity of the Rab8 antibody staining was confirmed by pre-incubation with recombinant Rab8 protein and by omitting the primary antibody. Bars, 200µm (A); 100µm (B), (C).

**Figure 2. Upregulation and targeting of Rab8 to the leading edge of macrophage foam cells.** (A) Western blot with anti-Rab8 monoclonal antibodies of primary human macrophages cultured in RPMI for 24h, loaded with 50µg/ml acLDL for 24h and subsequently incubated with 10µg/ml apoA-I for 18h. Equal sample loading was confirmed by Ponceau staining. (B) Filipin staining of primary human macrophages cultured in M-SFM + GM-CSF for 7 days and incubated with 50µg/ml acLDL and 10µg/ml apoA-I for 24h. (C) Quantification of macrophage elongation. Cells were incubated with 10µg/ml apoA-I, 50µg/ml acLDL, or apoA-I and acLDL together for 18h. The cells were stained with filipin, and the cell length vs. width ratio through the nucleus was determined. The five most elongated cells in each field were quantified, 10 fields in total, ***p<0.001. (D) Macrophages were incubated with acLDL and apoA-I as above and stained with filipin or anti-Rab8 antibodies. Arrowheads denote the lamellipodial area at the leading edge. Bars, 20µm (B), 10µm (D).

**Figure 3. Adenoviral overexpression of Rab8A upregulates ABCA1 and reduces cholesterol deposition in foam cells.** (A) Cell lysates from non-infected or infected cells were subjected to Western blotting using monoclonal anti-Rab8 antibodies. The myc-tagged Rab8A has a higher molecular weight than the endogenous Rab8. (B) Western blotting and quantification of ABCA1 levels in infected cells (duplicate determinations
from two independent donors, ***p<0.001). (C) Quantification of ABCA1 mRNA levels in infected cells (n=5, p=0.15). (D) Efflux of [³H]-cholesterol from infected macrophages to 10 µg/ml apoA-I (duplicate determinations from two independent donors, ** p<0.01). (E) Total cholesterol and (F) cholesteryl ester amounts in control and Rab8A infected cells (quadruplicate determinations from three independent donors, ** p<0.01, *** p<0.001).

Figure 4. Depletion of Rab8A in foam cells inhibits cholesterol efflux to apoA-I and increases deposition of acLDL-derived cholesterol. (A) Primary human macrophages were electroporated with control, Rab8A or Rab8b specific siRNAs. Knock-down efficiency was determined by Western blotting using monoclonal anti-Rab8 antibodies. (B) Macrophages were electroporated with control or Rab8A siRNA. The cells were loaded with 50 µg/ml acLDL for 24h and then incubated with 10 µg/ml apoA-I for 18h. Total cellular cholesterol content was measured from cell lysates after loading with acLDL and after efflux to apoA-I. Because the cholesterol content varied between individuals, the value after acLDL loading was set as 1.0 (duplicate determinations from three independent donors, * p<0.05). (C) Cells were electroporated as above and incubated with [³H]-Cholesteryl oleate labeled acLDL (50µg/ml) for 24h and apoA-I as above, followed by liquid scintillation counting (duplicate determinations from three independent donors, * p<0.05). (D) Efflux of the [³H]-cholesterol label from siRNA treated macrophages to 10 µg/ml apoA-I (duplicate determinations from two independent donors, ** p<0.01).
Figure 5. Rab8A depletion decreases the fraction of ABCA1 at the plasma membrane. (A) Control and Rab8A siRNA treated primary macrophages were subjected to surface biotinylation and anti-ABCA1 Western blotting. 1/10 of the sample was removed before pull-down of the biotinylated proteins to reflect the total cellular amount of ABCA1 (total). (B) Quantification of the fraction of ABCA1 on the cell surface in siRNA treated cells. Because the level of ABCA1 on the surface of control siRNA treated cells varied considerably between individuals (from 5 to 35% of total ABCA1), this value was set as 1.0 (n=6, independent donors, * p<0.05).
Supplementary Figure I

Colocalization between ABCA1 and Rab8 at plasma membrane protrusions.
Confocal section of HeLa cells expressing ABCA1-FLAG and Rab8-GFP. Cells were fixed at 48h post-transfection and stained with anti-FLAG antibodies and Alexa568 conjugated secondary antibodies.
Depletion of Rab8A from HeLa cells causes cholesterol accumulation and affects the intracellular routing of ABCA1-GFP.

(A) HeLa cells were treated with control or Rab8A siRNAs for three days, fixed and stained with anti-Rab8A antibodies. (B) HeLa cells were treated as above, loaded with 50µg/ml LDL during the last 18h, then fixed and stained with filipin. (C, D) Cells were treated with control or Rab8A siRNAs for two days after which the cells were transfected with ABCA1-GFP for 16h, fixed and stained with (C) anti-β1 integrin or (D) anti-transferrin receptor antibodies. Inset in the merged image shows a higher magnification of the area indicated. Bars, 10µm. The same focal plane in control and Rab8A siRNA treated cells is shown (for complete confocal stacks see Supplementary Figures III, IV).
Confocal stacks showing ABCA1-GFP and β1 integrin in HeLa cells treated with control or Rab8A siRNA.

HeLa cells treated with control or Rab8A siRNAs as indicated. After 48 h the cells were transfected with ABCA1-GFP for 16h, fixed and stained with anti-β1 integrin antibodies and Alexa568 conjugated secondary antibodies. Confocal stacks with ten Z sections per cell were taken. Z section 3 (indicated in blue) is shown in Supplementary Figure II C.
Supplementary Figure IV

Confocal stacks showing ABCA1-GFP and β1 integrin in HeLa cells treated with control or Rab8A siRNA.

HeLa cells treated with control or Rab8A siRNAs as indicated. After 48 h the cells were transfected with ABCA1-GFP for 16h, fixed and stained with anti-TfR antibodies and Alexa568 conjugated secondary antibodies. Confocal stacks with ten Z sections per cell were taken. Z section 4 (indicated in blue) is shown in Supplementary Figure II D.