Macrophage, But Not Systemic, Apolipoprotein E Is Necessary for Macrophage Reverse Cholesterol Transport In Vivo

Ilaria Zanotti, Matteo Pedrelli, Francesco Potì, Grazia Stomeo, Monica Gomaraschi, Laura Calabresi, Franco Bernini

**Objective**—To assess the role of apolipoprotein (apo) E in macrophage reverse cholesterol transport (RCT) in vivo.

**Methods and Results**—ApoE exerts an antiatherosclerotic activity by regulating lipoprotein metabolism and promoting cell cholesterol efflux. We discriminated between macrophage and systemic apoE contribution using an assay of macrophage RCT in mice. The complete absence of apoE lead to an overall impairment of the process and, similarly, the absence of apoE exclusively in macrophages resulted in the reduction of cholesterol mobilization from macrophages to plasma, liver, and feces. Conversely, expression of apoE in macrophages is sufficient to promote normal RCT even in apoE-deficient mice. The mechanisms accounting for these results were investigated by evaluating the first step of RCT (ie, cholesterol efflux from cells). Macrophages isolated from apoE-deficient mice showed a reduced ability to release cholesterol into the culture medium, whereas the apoB-depleted plasma from apoE-deficient and healthy mice possessed a similar capacity to promote cellular lipid release from cultured macrophages.

**Conclusion**—Our data demonstrate, for the first time to our knowledge, that apoE significantly contributes to macrophage RCT in vivo and that this role is fully attributable to apoE expressed in macrophages. (Arterioscler Thromb Vasc Biol. 2011;31:74-80.)

**Key Words:** ABC transporter ■ apolipoproteins ■ lipids ■ lipoproteins ■ macrophages ■ reverse cholesterol transport

Apolipoprotein E (apoE) is a structural component of several lipoproteins, including very-low-density lipoproteins (LDLs) and their remnants, chylomicron remnants, and high-density lipoproteins (HDLs). ApoE is predominantly synthesized by the liver and accomplishes its physiological role by driving the hepatic clearance of the lipoproteins on which it resides through binding with very LDL and chylomicron-remnant receptors and inhibiting triglyceride lipolysis. Altogether, these activities contribute to the regulation of circulating lipoprotein levels. Several population studies associated apoE defects with lipoprotein disorders and increased cardiovascular risk, thus revealing the key role played in atheroprotection. This beneficial activity was further demonstrated by the generation of mice lacking the apoE gene, characterized by hypercholesterolemia and abnormal lipid deposition in the proximal aorta and liver, even when receiving a normal chow diet. Beyond the influence on lipoprotein metabolism, apoE atheroprotective activity is also related to the promotion of cholesterol efflux from macrophages. Cholesterol efflux is the first rate-limiting step of reverse cholesterol transport (RCT), the process by which excess cholesterol is delivered from peripheral tissues to the liver for final excretion into the feces. Macrophage is the primary cell type overloaded with cholesterol within atherosclerotic lesions, and this cholesterol pool is the most important for atherosclerosis development and progression. Thus, the RCT that involves macrophage-derived cholesterol became fundamental concerning atheroprotection. Macrophage RCT can be estimated with a radioisotope-based assay, whose application has been useful for genetic and pharmacological investigations on this process. HDL are the major players of cholesterol efflux and RCT. HDL represent a heterogeneous class of particles whose composition influences the capacity to act as efficient lipid acceptors: large phospholipid-enriched HDL (α-HDL) specifically exchange lipids with scavenger receptor class B type I (SR-BI) and ATP-binding cassette G1 or through passive diffusion. Differently, small lipid-poor HDL (pre-β
HDL) preferentially bind to ATP-binding cassette A1 (ABCA1).20

The aim of this work was to investigate whether the antiatherosclerotic activity of apoE could be attributable to the promotion of macrophage RCT in vivo and to evaluate the contribution of systemic and macrophage apoE to this process.

Methods

The following materials were purchased commercially: cell culture media, FCS, PBS, BSA, the acyl-coenzyme A:cholesterol O-acyltransferase (ACAT) inhibitor Sandoz58035, probucol, organic solvents, [3H]-cholesterol, and tissue culture flasks and plates. 2-Hexyl-1-cyclopentanone thioglycollate (BLT-1) was a gift. Acetylated LDL (AcLDL) was prepared from human LDL, as previously described.21

Animals

C57BL/6 mice (wild type [WT]), aged 11 weeks, and apoE−/− (B6.129P2-apoEtm1Unc/Crl) mice (The Jackson Laboratory, Harbor, Me) received a standard chow diet (Mucedola) and water ad libitum. The study was performed with the approval of the Ethical Committee for Animal Experiments of the University of Parma, Parma, Italy.

Macrophage RCT In Vivo

Macrophage RCT in vivo was evaluated as previously described,12 using murine peritoneal macrophages (MPM) from WT or apoE−/− mice as cholesterol donors. After MPM harvesting from the peritoneum of thioglycollate-treated mice, cells were incubated for 48 hours with [3H]-cholesterol (5 μCi/mL) and AcLDL (25 μg/mL). In the first experiment (RCT 1), MPM from WT mice were intraperitoneally injected into WT mice (WT/WT) (n = 5), whereas macrophages from apoE−/− mice were injected into apoE−/− mice (apoE−/−/apoE−/−) (n = 5). In the second experiment (RCT 2), WT mice received MPM from WT mice (WT/WT) (n = 5) or apoE−/− mice (apoE−/−/WT) (n = 5). In the third experiment (RCT 3), both WT (WT/WT, n = 5) and apoE−/− mice (WT/apoE−/−, n = 5) were injected with WT macrophages. A full characterization of injected cells is provided (supplemental Figure I).

Lipid and Apolipoprotein Analysis

Plasma total cholesterol level was determined using a standard enzymatic technique with an autoanalyzer (Roche Diagnostics Integra 400). Plasma HDL cholesterol level was determined with the enzymatic technique with an autoanalyzer (Roche Diagnostics Instrumentation of thioglycollate-treated mice, cells were incubated for 48 hours. Cells were equilibrated in an albumin-containing medium for 18 hours, and lipid efflux was promoted to increasing concentrations of whole plasma from WT mice for 24 or 48 hours. Quantification of cholesterol efflux was performed using a time 0 set of cells to calculate total [3H]-cholesterol content in the monolayer, as previously described.24 Fractional efflux is calculated as follows: counts per minute of [3H] in the medium/(μg [3H] at time 0 × 100).

Evaluation of HDL Efflux Potential

The capacity of HDL from WT or apoE−/− mice to promote in vitro cellular cholesterol efflux was evaluated in cholesterol-enriched WT MPM, with a standard lipid efflux assay as previously described. In the equilibration period, some cells were treated with probucol (10 μmol/L) or BLT-1 (1 μmol/L) to inhibit ABCA125 or SR-BI26 activity, respectively. Cholesterol release was promoted to plasma diluted to 0.1% to 0.5% to 1% after removal of apoB-containing lipoproteins. This HDL fraction was obtained from whole plasma by dextran sulfate–magnesium chloride precipitation, as previously described and is referred in the text as HDL.

2D Gel Electrophoresis

HDL subclasses were separated by 2D electrophoresis, in which agarose gel electrophoresis was followed by nondenaturing polyacrylamide gradient gel electrophoresis and subsequent immunodetection with anti–mouse apoA-I. The relative content of pre-β HDL was calculated using computer software (Bio-Rad Multi-Analyzer/PC Software) and expressed as percentage of total apoA-I.

Statistical Analysis

Data are reported as mean±SD. Results were analyzed by the 2-tailed Student t test and a 2-way ANOVA, followed by post hoc analysis using a Bonferroni test with the use of computer software (GraphPad Prism Software).

Results

Macrophage RCT Is Impaired in ApoE−/− Mice

To evaluate the role of apoE in the antiatherosclerotic process of RCT in vivo, we assessed the mobilization of radiolabeled cholesterol from macrophages injected into the peritoneum of apoE−/− or WT mice to plasma, liver, and feces by a method that has been widely applied to physiological and pharmacological studies.12–16 The mice used in our experiments presented the typical plasma lipid profile described for these strains:6–7: the deletion of apoE produced a 6.4-fold increase of total cholesterol and a 76% decrease of HDL cholesterol levels compared with WT animals, whereas apoA-I content was diminished by 41% (Figure 1). The injection of either WT or apoE−/− macrophages for the RCT measure did not affect this phenotype (Figure 1). In addition, a Western blot analysis demonstrated that no detectable levels of apoE were present in the plasma of apoE−/− mice injected with WT macrophages (supplemental Figure I).

As a first approach, [3H]-cholesterol–loaded macrophages from apoE−/− mice were injected into apoE−/− mice (apoE−/−/apoE−/−), whereas WT macrophages were injected into WT mice (WT/WT). At 48 hours after the injection of macrophages, animals were euthanized and samples of blood, liver,
and feces were collected for analysis. The amount of radioactivity in plasma, liver, and feces was quantified to evaluate cholesterol distribution along the RCT pathway. Compared with WT/WT animals, apoE+/H11002+/H11002/+/apoE+/H11002+/H11002/+/apoE+/H11002+/H11002 mice exhibited a slight increase in plasma [3H]-cholesterol (Figure 2A), but reduction of hepatic (Figure 2B) and fecal (Figure 2C) [3H]-cholesterol levels, by 40% and 43%, respectively. Overall, cholesterol mobilized from macrophages, calculated as the sum of radioactivity detected in plasma, liver, and feces, was 26% lower in apoE+/H11002+/H11002/+/apoE+/H11002+/H11002/+/apoE+/H11002+/H11002 mice (Figure 2D).

Macrophage ApoE Affects Cholesterol Efflux and Macrophage RCT In Vivo

The first rate-limiting step of the RCT pathway, cholesterol efflux from macrophages into the plasma compartment, can be influenced by 2 factors: (1) cell capacity to release cholesterol to plasma and (2) HDL capacity to act as a lipid acceptor.18 To investigate the role of apoE in this process, we compared the capacity of macrophages from WT and apoE+/H11002+/H11002 mice to release cholesterol and the capacity of HDL from WT and apoE+/H11002+/H11002 mice to act as a lipid acceptor. Cell cholesterol efflux was assessed by labeling MPM from WT and apoE+/H11002+/H11002 mice with [3H]-cholesterol and exposing them to increasing concentrations of whole plasma for 24 hours. As documented by Figure 3, apoE-deficient cells showed a severe reduction of lipid release, both in the presence and absence of an extracellular acceptor. The same result was obtained in cells exposed to plasma for 48 hours (data not shown). This observation suggests that apoE specifically expressed in macrophages is necessary for efficient cholesterol efflux. To assess the impact of apoE-mediated macrophage efflux on RCT in vivo, the process was evaluated in healthy mice receiving apoE+/H11002+/H11002 macrophages (apoE+/H11002+/H11002/+/WT), and the extent of radiolabeled cholesterol mobilization was compared with that in WT/WT mice. The lack of apoE specifically in macrophages caused a significant reduction of [3H]-cholesterol content in plasma (~51%, Figure 4A), liver (~17%, Figure 4B), and feces (~25%, Figure 4C). Overall, the total amount of [3H]-cholesterol mobilized from macrophages was reduced by 29% (Figure 4D). Thus, apoE deletion...
exclusively in macrophages is sufficient to impair macrophage RCT.

**Systemic ApoE Deletion Does Not Significantly Affect Macrophage RCT**

HDL capacity to act as a lipid acceptor was measured by quantifying cholesterol efflux from cholesterol-enriched MPM treated or not treated with probucol and BLT-1 to investigate the specific contributions of ABCA1 and SR-BI, respectively. In this experiment, whole plasma derived from WT and apoE−/− mice was previously treated with dextran sulfate–magnesium chloride to isolate the HDL fraction and exposed to cells at increasing concentrations. The data displayed in Figure 5A indicate that HDL from apoE−/− animals possessed a similar to slightly higher capacity to drive cholesterol efflux. This result is attributable to a larger contribution from ABCA1, as demonstrated by the larger reduction of cholesterol efflux in the presence of probucol compared with WT HDL (55% versus 31% to apoE−/− and WT HDL, respectively) (Figure 5B). The treatment with BLT-1 produced no difference, consistent with a little impact of SR-BI in mediating cholesterol release from cholesterol-loaded macrophages. The role of ABCA1 in mediating cholesterol efflux to apoE−/− HDL was confirmed in J774 treated or not treated with 8-(4-chlorophenylthio)–cAMP to upregulate transporter expression. In this cell model, ABCA1-mediated efflux was significantly higher (29%) to apoE−/− HDL compared with WT HDL (supplemental Figure II). The increase in the process that occurred via ABCA1 suggests that the severe reduction of HDL observed in apoE−/− mice did not involve those particles responsible for the interaction with ABCA1. To validate this hypothesis, 2D gel electrophoresis was performed, revealing that HDL from apoE−/− mice possessed an intact pre-β HDL portion (% of pre-β on total HDL: 35.0%±8.2% and 13.9%±2.8% in WT and apoE−/− mice, respectively; n=3), whereas the larger α HDL portion was significantly reduced (Figure 6). To assess the impact of HDL efflux potential on RCT in vivo, the process was evaluated in apoE−/− mice receiving WT macrophages (WT/apoE−/−) and the extent of radioabeled cholesterol mobilization was compared with that in WT/WT mice. As shown in Figure 7A, the lack of systemic apoE caused a 2.5-fold increase of [3H]-cholesterol content in plasma.
plasma 48 hours after the injection of cholesterol-loaded WT macrophages. The amount of $[^{3}H]$-cholesterol in the liver and feces was not significantly different between the 2 groups (Figure 7B and 7C, respectively), whereas the total amount of $[^{3}H]$-cholesterol mobilized from macrophages was even higher in WT/apoE$^{-/-}$/apoE$^{-/-}$ mice (Figure 7D).

**Discussion**

The pivotal role of apoE in cholesterol homeostasis is well exemplified by the appearance of hypercholesterolemia and diffuse atherosclerosis in humans or animals carrying apoE defects$^{5-7}$ and is mostly attributed to the influence on lipoprotein metabolism$^3$ and the capacity to promote cholesterol efflux from cells.$^{8,9,29}$ Although the former effect is related to systemic apoE, which mediates lipoprotein clearance and regulates HDL plasma levels, the latter effect is mainly exerted by apoE specifically expressed in macrophages. Both of these effects could result in a positive impact on RCT, but no direct evidence of apoE contribution to this process in vivo is provided. In this study, we aimed to assess the impact of systemic and macrophage apoE through the evaluation of macrophage RCT in vivo and established that macrophage, but not systemic, apoE is relevant for functional macrophage RCT in vivo. The process extent was first compared in apoE$^{-/-}$ mice receiving apoE$^{-/-}$ macrophages and in WT mice receiving WT macrophages to investigate the impact of the complete absence of this apolipoprotein. RCT was severely impaired in apoE$^{-/-}$/apoE$^{-/-}$ mice, as evidenced by the reduction of $[^{3}H]$-cholesterol in liver and feces. On the contrary, the level of $[^{3}H]$-cholesterol in plasma is higher in apoE$^{-/-}$/apoE$^{-/-}$ mice, possibly because of the increased retention of tracer because of the enormous pool of plasma cholesterol typical of apoE$^{-/-}$ mice. This explanation is supported by previous reports using this experimental method, in which the levels of $[^{3}H]$-cholesterol tracked with cholesterol mass.$^{23}$ However, the sum of radioactivity in plasma, liver, and feces was lower in apoE$^{-/-}$/apoE$^{-/-}$ mice, confirming the reduced efficiency of cholesterol mobilization from macrophages in these animals. Macrophage RCT reflects the removal of cholesterol from the arterial wall, and its impairment is associated with increased atherosclerosis in several animal models.$^{23,30-32}$ Our present findings propose a mechanism by which mice with the complete deletion of the apoE gene develop spontaneous arterial lesions.$^6,7$

Cholesterol efflux from cells is considered the first rate-limiting step of RCT$^{33}$ and is influenced by either the ability of cells to release cholesterol and the capacity of plasma HDL to act as an extracellular acceptor.$^{18}$ First, we compared the capacity of thioglycollate-elicited macrophages from WT or apoE$^{-/-}$ mice to release cholesterol to whole mouse plasma and observed a defective ability from apoE$^{-/-}$ cells. The contribution of endogenous apoE in promoting lipid efflux

![Figure 7](http://atvb.ahajournals.org/)

**Figure 7.** Systemic ApoE deficiency does not affect macrophage RCT in vivo. A through C, Macrophage-derived $[^{3}H]$-cholesterol was quantified in plasma (A), liver (B), and feces (C). Quantification of radioactivity content was performed as described for Figure 2. D, Total RCT was calculated as the sum of radioactivity detected in plasma, liver, and feces. Results are expressed as percentage of injected $[^{3}H]$ dose±SD (mean±SD) (n=5 mice per group). *$P<0.05$ and **$P<0.01$ vs WT/WT mice.
from macrophages to apoA-I or HDL was previously documented.8,34 Our data confirm these observations in a more physiological setting, represented by the whole plasma. Even in the absence of extracellular acceptors, apoE−/− macrophages effluxed significantly less cholesterol than WT macrophages. This result is consistent with a report15 showing that cholesterol release from apoE−/− macrophages to cell medium alone is impaired. Therefore, it is conceivable that the synthesis and secretion of apoE from macrophages is sufficient to promote cholesterol release, even in the absence of extracellular acceptors. An apoE-dependent increase in cholesterol efflux suggests that apoE selectively expressed in macrophages may efficiently improve RCT in vivo by enhancing cholesterol availability for transport to liver and feces. To confirm this hypothesis, we measured RCT in WT mice receiving either apoE−/− or WT macrophages and demonstrated that the overall process was defective in apoE−/−/WT mice. In this experiment, the reduced macrophage-derived cholesterol in plasma, liver, and feces indicates that apoE deletion in macrophages is sufficient to impair the whole RCT. In agreement with this observation, increased atherosclerosis was observed in healthy mice reconstituted with apoE−/− macrophages. This result is consistent with a report35 showing that cholesterol release from apoE−/− macrophages is not related to the absence of systemic apoE but rather to its specific deletion in macrophages. This result to human physiology and pathology. In fact, in contrast to humans, mice do not express the cholesteryl ester transfer protein (CETP), resulting in modifications of the RCT pathway. In fact, although in the CETP-deficient species, the transfer of cholesterol to the liver is mainly mediated by apoE-enriched HDL, in CETP-expressing species most cholesterol is absorbed by the liver after transfer to apoB-containing lipoproteins.39 However, our data demonstrating the role of apoE specifically in macrophages describe a CETP-independent mechanism and are, therefore, poorly influenced by the lack of this protein.

In conclusion, our data demonstrate, for the first time to our knowledge, the following: (1) apoE plays a relevant role in driving macrophage RCT in vivo because its complete deletion is associated with a 30% decrease of the process and (2) this effect is fully related to the expression of apoE in macrophages, promoting cell cholesterol efflux. A systemic effect can be ruled out by the demonstration that injection of apoE-expressing macrophages in apoE−/− mice does not affect plasma lipid profile.

Similar to our observation, Wang and colleagues32 demonstrated that ABCA1 and ABCG1 deletion in macrophages reduced macrophage RCT extent by 30%.

Although the present study clearly demonstrates the significant contribution of apoE in the macrophage RCT, J774 cells (which do not express apoE) are often used in RCT experiments of this type,2,12,13,15 thus indicating that even in the absence of this component, a substantial efflux still occurs. In addition, an important concept emerging from the present and other studies is that cholesterol efflux and macrophage RCT efficiency may be predictors of atherosclerosis extent: when the former is increased, the latter is decreased (and vice versa).10 Our study fully confirms this inverse correlation, thus providing a mechanism for the impact of macrophage apoE on atherosclerosis demonstrated with the bone marrow transplantation technique on mice.36–38

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Disclosures

None.

References


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# Supplemental Table I. Characterization of injected MPM

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<th>RCT 1</th>
<th>Cpm/mouse</th>
<th>n°cells/mouse</th>
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<td><strong>WT/WT</strong></td>
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<td>RCT 2</td>
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<td><strong>WT/ APOE^-/-</strong></td>
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<td>18.76×10^6</td>
</tr>
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Supplemental Figure IS

Blood samples were collected into K-EDTA tubes by retro-orbital bleeding 48h after cell injection. Plasma was isolated by low speed centrifugation (3000 x g, 20 minutes at 4° C). 3 µl of mouse plasma under non-reducing and heating conditions was loaded per lane on SDS-PAGE 12 % gels and separated for 1 h and 30 min at 30 mA/gel. Gels were transferred to nitrocellulose membranes for 1 h at 90 V and the transfer quality was evaluated by Ponceau red staining. Membranes were incubated in TBST buffer (1 x TBS [pH 7.4] and 0.1 % Tween 20) with 5% low fat milk for 1 h at room temperature before addition of the primary antibody. The primary antibody was diluted (1:500) in TBST buffer - 1 % low fat milk and incubated with membranes overnight at 4°C. Membranes were washed 3 x 10 min with TBST buffer. As a primary antibody a rabbit anti-mouse apoE (Biodesign K23100R) was used. Horseradish peroxidase–conjugated donkey anti-rabbit IgG (GE Healthcare, USA) were diluted 15.000 fold in TBST - 1 % low fat milk and incubated with membranes for 30 min at room temperature. Membranes were washed 3 x 10 min with TBST and visualised using chemiluminescence assay (ECL Plus, GE Healthcare, USA).

‘+’ positive control (WT mouse); ‘-’ negative control (apoE<sup>−/−</sup> mouse); ‘1 to 6’ WT/apoE<sup>−/−</sup> mice.
J774 macrophages were radiolabeled with $[^3]$H-cholesterol 2µCi/ml in presence of the ACAT inhibitor 2µg/ml for 24h and equilibrated in an albumin containing medium containing or not cpt-cAMP 0.3mM for 18h, before the incubation for 6h with apoB-depleted plasma from WT or apoE$^{/-}$ mice diluted to 1%. ABCA1 contribution was calculated as the difference between fractional efflux in cells treated with cpt-cAMP and in basal condition. The experiment was carried out in triplicates, testing 3 samples of HDL/group. Efflux was expressed as cpm in medium/cpm To*100 ± SD

*p<0.05 vs WT HDL.