Reverse Cholesterol Transport Is Increased in Germ-Free Mice—Brief Report

Rima H. Mistry, Henkjan J. Verkade, Uwe J.F. Tietge

Objective—The intestinal microbiota is emerging as a clinically relevant modulator of atherosclerotic risk. Reverse cholesterol transport (RCT) is an atheroprotective metabolic pathway. How the microbiota impacts RCT has not been investigated. Therefore, the aim of this study was to characterize (cholesterol) metabolism and RCT in germ-free mice compared with conventional mice.

Approach and Results—In chow-fed germ-free mice, plasma cholesterol was unchanged, whereas liver cholesterol content was higher (1.5-fold; $P<0.05$) than in conventional controls. Biliary secretion of cholesterol (2-fold; $P<0.001$) and bile acids (3-fold; $P<0.001$) was substantially increased in the germ-free model, whereas fecal neutral sterol excretion was unaltered, and fecal bile acid excretion was decreased ($P<0.01$). However, fecal bile acid profiles of germ-free mice were dominated by the presence of β-muricholic acid ($P<0.001$), pointing toward a higher contribution of the alternative acidic pathway to total bile acid synthesis in these mice. As expected, secondary bile acids were absent in the germ-free model. In vivo macrophage-to-feces RCT was increased >2-fold ($P<0.01$) in the absence of intestinal bacteria.

Conclusions—These data demonstrate that the absence of the intestinal microbiota stimulates RCT >2-fold. Thereby, our results support the importance of intestinal bacteria for metabolic regulation and indicate that specific targeting of the microbiota bears therapeutic potential to prevent and treat cardiovascular disease.

Visual Overview—An online visual overview is available for this article. **(*Arterioscler Thromb Vasc Biol*. 2017;37:419-422. DOI: 10.1161/ATVBAHA.116.308306.)**

Key Words: bacteria ■ cholesterol ■ liver ■ microbiota ■ sterols

Evidence is accumulating that the intestinal microbiota has a substantial impact on the (patho)physiological regulation of metabolism. The human microbiota in general represents not only the first line of contact with the environment, but intestinal bacteria in particular also express ≈100-fold more genes than present in the human genome.[1] The intestine is one of the key organs in the regulation of cholesterol metabolism with relevance for atherosclerotic cardiovascular disease. Enterocytes are responsible for cholesterol absorption, can synthesize cholesterol, and form high-density lipoprotein particles.[2] As a precedence for the impact of bacteria on cardiovascular disease, it was demonstrated that specific diet–microbe–host interactions can enhance experimental atherosclerosis via the coordinate production of the proatherosclerotic metabolite trimethylamine-N-oxide.[3] Subsequently, trimethylamine-N-oxide was also identified as a prospective biomarker for the future development of cardiovascular disease events in the general population, further stressing the relevance of the microbiota for human disease.[4] In addition, bacteria can modulate bile acid (BA) metabolism and thereby impact cholesterol turnover.[5] In conventional mice, the intestine was shown to contribute to reverse cholesterol transport (RCT), a key atheroprotective pathway.[6] However, the impact of the intestinal microbiota per se on RCT has not been determined. Therefore, the aim of the present work was to establish the importance of the intestinal microbiota for RCT by comparing conventional with germ-free mice.

See accompanying editorial on page 385

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

First, we characterized cholesterol metabolism in germ-free mice compared with conventional controls. Both groups had similar body weights (21.3±1.1 versus 21.4±1.5 g). Although plasma total cholesterol (Figure [A]) and triglyceride levels (0.26±0.02 versus 0.28±0.02 mmol/L) were comparable between groups, FPLC profiles showed a discrete shift toward higher low-density lipoprotein-cholesterol and lower high-density lipoprotein-cholesterol in germ-free animals (Figure IA in the online-only Data Supplement). High-density lipoprotein cholesterol efflux capacity did not differ between groups (Figure IB in the online-only Data Supplement). Hepatic cholesterol content was higher in germ-free mice by 1.5-fold (5.90±1.25

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Nonstandard Abbreviations and Acronyms	
Fgf15	fibroblast growth factor 15
FXR	farnesoid X receptor
RCT	reverse cholesterol transport
MCA	muricholic acid

versus 4.23±0.73 nmol/mg; $P<0.05$) and in agreement with these data, expression of the sterol regulatory element-binding protein (*Srebp2)* target genes low-density lipoprotein receptor (*Ldlr*; Table; $P<0.001$) and HMG-CoA reductase (*Hmg-Coar*; Table; $P<0.05$) was decreased. Biliary cholesterol and bile acid secretion was ≈2- and 3-fold increased, respectively, in mice lacking microbiota ($P<0.001$; Figure [B] and [C]). Fecal output was significantly increased in germ-free mice (213±26 versus 162±12 mg per day; $P<0.05$). However, fecal excretion of neutral sterols remained unchanged, whereas bile acid excretion was reduced by 1.5-fold (Figure [D]; $P<0.01$). Increased mRNA expression of Niemann-Pick C1-like1 (*Npc1l1)* in the proximal small intestine (Table; $P<0.05$) indicated increased cholesterol absorption in germ-free mice.

Next, a RCT experiment was performed. Plasma ^{3}H-cholesterol tracer recovery was significantly increased by ≈2-fold in germ-free mice after 24 hours ($P<0.01$; Figure [E]) and tended to be higher at 48 hours. Tracer recovery in the liver at 48 hours remained unchanged (Figure [F]). Overall RCT, determined as fecal recovery of macrophage-derived ^{3}H-cholesterol, was 2-fold higher in germ-free mice (Figure [G]; $P<0.01$). This increase was largely because of significantly more tracer recovered in fecal bile acids ($P<0.01$). Stress could potentially explain these findings,[7] but plasma corticosterone levels were comparable between conventional and germ-free mice making this possibility less likely (1466±219 versus 1496±159 nmol/L, respectively).

Therefore, we also characterized bile acid metabolism under germ-free conditions more in detail. Bile flow was significantly increased compared with conventional mice (2-fold; $P<0.001$; Figure IIA in the online-only Data Supplement). Although fecal mass excretion of bile acids was lowered, there were striking shifts in the fecal bile acid profile in germ-free mice. As expected, secondary bile acids such as deoxycholic acid and ω-muricholic acid (ω-MCA) were absent. On the other hand, the percentage of fecal cholic acid was higher ($P<0.05$), and the contribution of β-muricholic acid (β-MCA) was increased by 4.5-fold in mice lacking microbiota ($P<0.001$; Figure IIA and IIB in the online-only Data Supplement). Quantification of hepatic bile acid synthesis gene expression (Table) revealed decreased *Cyp8b1* ($P<0.01$) and *Cyp7a1* ($P<0.001$) mRNA levels in germ-free

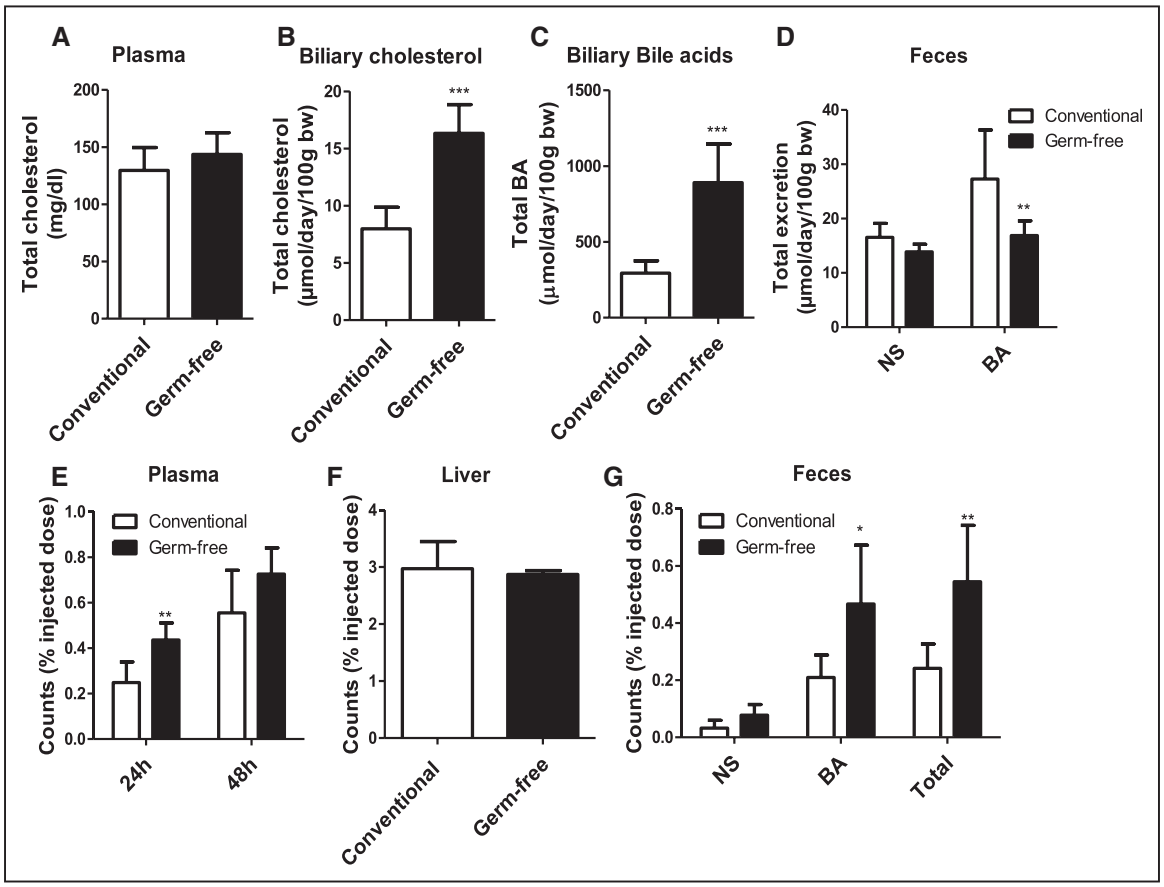

Figure. Absence of intestinal microbiota stimulates macrophage-to-feces reverse cholesterol transport (RCT). Mass measurements (**A**) Plasma total cholesterol; (**B**) biliary cholesterol secretion; (**C**) total biliary bile acid (BA) secretion; (**D**) fecal mass neutral sterol (NS) and bile acid excretion; RCT experiment, macrophage-derived cholesterol tracer recovered in (**E**) plasma; (**F**) liver; (**G**) fecal neutral sterols and bile acids. Data are presented as means±SD, n=7 for each group. Statistically significant differences are indicated as *$P<0.05$; **$P<0.01$, ***$P<0.001$.

Table. Gene Expression Analysis in Conventional and Germ-Free Mice

Genes	Conventional	Germ Free
Liver		
Hmgcoar	1.00±0.08	0.83±0.05*
Cyp7a1	1.00±0.42	0.26±0.14†
Cyp8b1	1.00±0.29	0.36±0.15‡
Cyp27a1	1.00±0.15	0.84±0.13
Abcg5	1.00±0.19	1.06±0.08
Abcg8	1.00±0.27	1.18±0.06
Bsep	1.00±0.25	0.75±0.24*
Srb1	1.00±0.11	0.95±0.15
Fxr	1.00±0.26	0.89±0.19
Ldlr	1.00±0.12	0.68±0.07†
Proximal intestine		
Npc1l1	1.00±0.09	1.75±0.08*
Distal intestine		
Fgf15	1.00±0.15	2.61±0.30*

Data are presented as means±SD, n=7 for each group.
*P<0.05, statistically significant difference.
†P<0.001, statistically significant difference.
‡P<0.01, statistically significant difference.

mice, whereas *Cyp27a1*, which initiates the alternative acidic pathway with β-MCA as end product, remained unchanged. mRNA expression of *fibroblast growth factor 15 (Fgf15)* in the terminal ileum was 2.6-fold increased in the germ-free model (Table; P<0.05).

Discussion

The results of this study demonstrate that complete absence of the microbiota (1) does not influence plasma cholesterol levels or mass fecal neutral sterol excretion, (2) decreases fecal BA excretion, and (3) significantly increases RCT, mainly within the fecal BA fraction. Previous work indicated that differences in cholesterol metabolism between germ-free and conventional mouse models are variable; the observed phenotypes conceivably depend on diet, genetic background, and the respective composition of the microbiota in the conventional control groups.[8–10] Thus far, decreased plasma cholesterol, both increased and decreased liver cholesterol, and higher fecal neutral sterol output were observed in germ-free mice fed western-type or high-fat diets.[10,11] Interestingly though, the most significant differences between conventional and germ-free mice in our experimental system were seen in bile acids.

The changes in BA metabolism occurring in the germ-free mice are, however, rather complex; on the one hand, there is increased biliary BA secretion, on the other decreased fecal excretion. This difference can be explained by increased BA reabsorption in the terminal ileum of germ-free mice, a notion in general consistent with the increased expression of the farnesoid X receptor (FXR) target gene *Fgf15* that we observed in our study. However, the 2.6-fold increase in *Fgf15* expression in the germ-free model also indicates another relevant change in BA metabolism in these mice. Mice lacking intestinal bacteria have 2 principal BA species, the more hydrophobic taurocholic acid, which is an FXR agonist,[12] and the hydrophilic tauro-β-muricholic acid (T-β-MCA), which has been characterized as an FXR antagonist.[13,14] Increased expression of the FXR target gene *Fgf15* indicates thus that relatively more taurocholic acid, the FXR agonist, is taken up over T-β-MCA, the FXR antagonist. Indeed, previous work demonstrated a substantially higher affinity of ASBT, the apical transporter responsible for intestinal BA reuptake, for taurocholic acid compared with T-β-MCA.[15] Since due to the absence of bacteria no secondary BA are formed in germ-free mice, there is no means of taking up conversion products of T-β-MCA in the colon, thus resulting in substantial amounts of T-β-MCA being excreted into the feces. In agreement, hepatic gene expression analysis indicated that the expression of Cyp27a1 is unchanged, pointing toward a higher relative contribution of the alternative acidic bile acid synthesis pathway with its end product T-β-MCA in the germ-free mice. It is thus to be expected that in the absence of intestinal bacteria relatively more macrophage-derived cholesterol is converted into T-β-MCA, which is then preferentially excreted into the feces. These mechanisms can in our view explain how increased RCT mainly in the BA fraction occurs in germ-free mice with decreased mass fecal BA excretion. However, more experimentation seems required to fully substantiate this proposed model.

Further, it has to be noted that converting chenodeoxycholic acid into β-MCA occurs in mice but not in humans.[16] Therefore, further studies are needed to investigate whether the results of our current study can be translated to a clinical setting in humans. In addition, also about bile acid metabolism in germ-free mice, variable data have been generated, likely dependent on genetic background and diet, for example, unchanged and decreased fecal bile acid excretion were reported.[8,14] Clearly, also with respect to this issue, more studies are needed to better characterize the response of different germ-free mouse lines to varying experimental conditions. In summary, our present work supports the importance of the intestinal microbiota for metabolic regulation and extends previous observations to RCT, a pathway with a high relevance for atherosclerosis protection. Specific targeting of the intestinal microbiota with the aim to modulate bile acid metabolism bears therapeutic potential with the goal to prevent and treat cardiovascular disease.

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Disclosures

None.

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Highlights

- This study characterized sterol metabolism and reverse cholesterol transport in conventional versus germ-free mice.
- Biliary secretion of cholesterol and bile acids increased substantially in germ-free mice.
- Germ-free mice had unchanged fecal excretion of neutral sterols but lower bile acid excretion.
- Reverse cholesterol transport was >2-fold higher in the absence of intestinal bacteria.
- Specific targeting of the microbiota bears therapeutic potential to prevent and treat cardiovascular disease.

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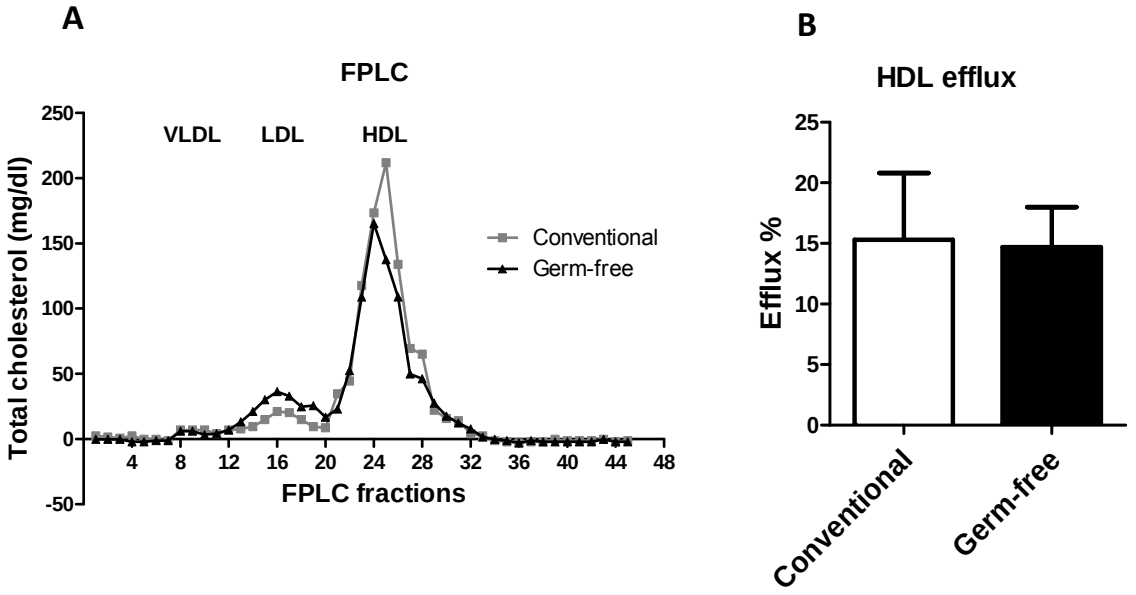
SUPPLEMENTARY FIGURES

Mistry RH, et al. " Reverse cholesterol transport is increased in germ-free mice"

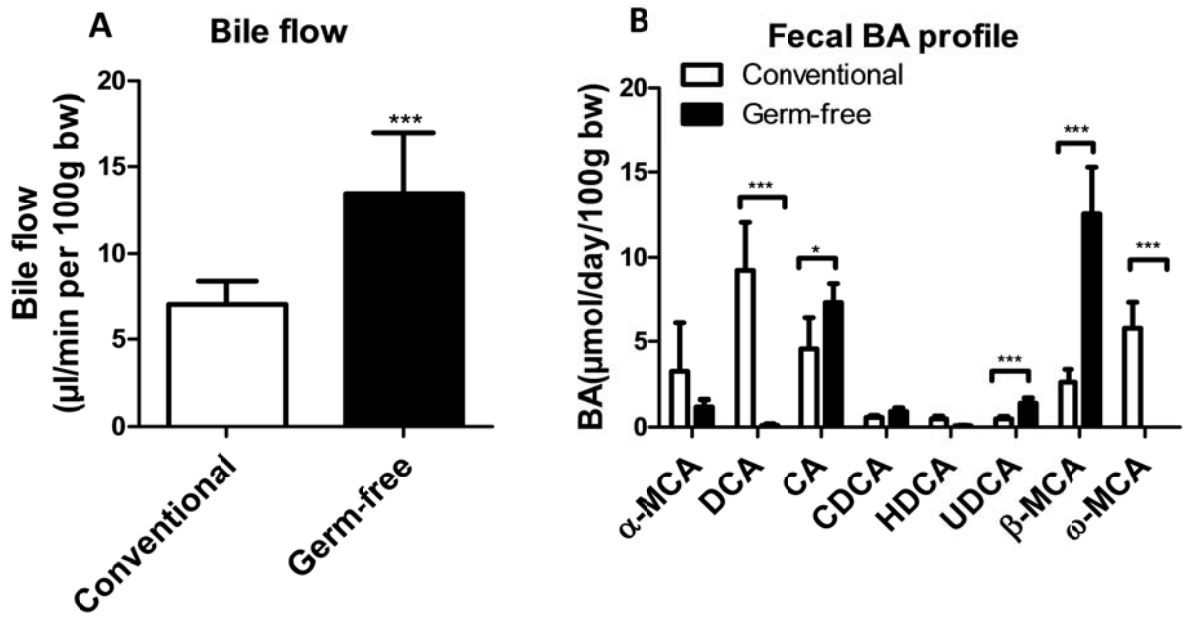
Figure legends

Supplementary Figure I: FPLC profiles (A) and HDL cholesterol efflux (B) in conventional and germ-free mice. Data are presented as means ± SD, N=7 for each group.

Supplementary Figure II: Bile flow (A) and fecal bile acid profiles (B) in germ-free and conventional mice.. α-MCA, α-muricholic acid; DCA, deoxycholic acid; CA, cholic acid; CDCA, chenodeoxycholic acid; HDCA, hyodeoxycholic acid; UDCA, ursodeoxycholic acid; β-MCA, β-muricholic acid; ω-MCA, ω-muricholic acid. Data are presented as means ± SD. N=7 for each group. Statistically significant differences are indicated as *P<0.05; **P<0.01, ***P<0.001.



Supplementary Figure I



Supplementary Figure II

SUPPLEMENTAL MATERIALS AND METHODS

Mistry RH, et al. " Reverse cholesterol transport is increased in germ-free mice"

Materials and Methods

Experimental animals
Wildtype conventional C57BL/6 OlaHsd mice were obtained from Harlan (Horst, The Netherlands). Wildtype female germ-free C57BL/6 mice were generated by cesarean section and foster nursing with germ-free Swiss-Webster mice (Taconic, Ejby, Denmark) in our animal facility and maintained in sterile flexible gnotobiotic isolators. At least seven mice were used per group for all experiments. All animals were housed in controlled rooms with an alternating 12h light-dark cycle. Mice were fed standard chow diet (Ssniff, Germany) sterilized with γ-irradiation (50 kGy). All experiments were approved by the Committee of Animal Experimentation at the University of Groningen and performed in accordance with the Dutch National Law on Animal Experimentation and international guidelines on animal experimentation.

Plasma and liver lipid analyses and determination of corticosterone levels
At the time of termination, liver and blood were collected by heart puncture. Plasma total cholesterol and triglycerides were measured using commercially available reagents (Roche Diagnostic, Basel, Switzerland). Pooled plasma of each group was subjected to fast protein liquid chromatography (FPLC) gel filtration using a Superose 6 column (GE Health, Uppsala, Sweden) essentially as described previously.[1] Livers were homogenized and lipids extracted following the general procedure described by Bligh & Dyer.[2] Lipids were redissolved in water containing 2% Triton X-100. Total cholesterol was measured as detailed above. Plasma corticosterone levels were determined using liquid-chromatography mass spectrometry with corticosterone-d4 as internal standard.

Bile measurements
Continuous bile cannulation was performed under anesthesia (hypnorm 1 ml/kg body weight; diazepam 10 mg/kg body weight) for 20 minutes. Biliary bile acid concentrations were determined using liquid-chromatography mass spectrometry and cholesterol concentrations were determined using commercially available reagents (Roche Diagnostic, Basel, Switzerland). The biliary secretion rates are calculated as described previously.[1]

Fecal sterol and bile acid analysis
Fecal samples from individually housed mice collected over 24 hours were dried, weighed and ground. Neutral sterols and bile acids were extracted and measured using gas-liquid chromatography as published.[1]

Macrophage-to-feces RCT studies
C57BL/6 donor mice were used to harvest primary peritoneal thioglycollate-elicited macrophages.[3] Macrophages were loaded *in vitro* with 5μg/ml acetylated LDL and 3μCi/ml [^{3}H] cholesterol (Perkin Elmer, Boston, MA) for 24 hours to become foam cells.[3] After equilibration with RPMI 1640 medium supplemented with 2% BSA (Sigma) for 18 hours macrophages were injected subcutaneously into individually housed recipient mice. At indicated time points plasma, liver and fecal recovery of labeled cholesterol was analyzed using liquid scintillation counting

(Packard 1600CA Tri-carb, Packard, Meriden, CT). For this purpose a piece of liver was solubilized in Solvable (Packard).[4] Fecal neutral sterol and bile acid fractions were extracted from feces as described above.[2] Fecal counts are calculated for total fecal output. The counts were expressed relative to the injected dose.

Hepatic gene expression analysis
Hepatic mRNA was extracted using TriReagent (Sigma) and quantified with a Nanodrop ND-100UV-vis spectrometer (NanoDrop Technologies Wilmington DE). cDNA was made from 1µg of RNA using reagents from Invitrogen (Carlsbad CA). Primers were synthesized by Eurogentec (Seraing, Belgium). Real-time PCR was performed using an ABI Prism 7700 machine (Applied Biosystems, Damstadt Germany). mRNA expression levels of individual genes were calculated relative to the housekeeping gene *36B4* and normalized to the relative mean expression level of the respective control group.

In vitro macrophage HDL efflux assay
Primary thioglycollate-elicited peritoneal macrophages were harvested from C57BL/6 mice, cultured and loaded to become foam cells as detailed above. Then cells were incubated with RPMI 1640 supplemented with 2% apoB-depleted mouse plasma.[5] After 5 hours of efflux, medium was collected and centrifuged for 5 minutes at 10,000 rpm. 0.1M NaOH was added to the cells and incubated for at least 30 minutes. The radioactivity in both medium and cells was determined using liquid scintillation counting (Packard 1600CA Tri-carb, Packard, Meriden, CT). Cholesterol efflux was calculated as follows: percent efflux = [counts in medium / counts in cells + counts in medium]*100%.[5] Values of negative control wells without added HDL were subtracted from all experimental data.

Statistics
Statistical analysis was performed using GraphPad Prism software (San Diego, CA). All data are presented as mean ± SD. Differences between groups were determined using the Mann-Whitney U-test. P-values below 0.05 were considered statistically significant.

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