Transintestinal and Biliary Cholesterol Secretion Both Contribute to Macrophage Reverse Cholesterol Transport in Rats—Brief Report

Jan Freark de Boer, Marleen Schonewille, Arne Dikkers, Martijn Koehorst, Rick Havinga, Folkert Kuipers, Uwe J.F. Tietge, Albert K. Groen

Objective—Reverse cholesterol transport comprises efflux of cholesterol from macrophages and its subsequent removal from the body with the feces and thereby protects against formation of atherosclerotic plaques. Because of lack of suitable animal models that allow for evaluation of the respective contributions of biliary cholesterol secretion and transintestinal cholesterol excretion (TICE) to macrophage reverse cholesterol transport under physiological conditions, the relative importance of both pathways in this process has remained controversial.

Approach and Results—To separate cholesterol traffic via the biliary route from TICE, bile flow was mutually diverted between rats, continuously, for 3 days. Groups of 2 weight-matched rats were designated as a pair, and both rats were equipped with cannulas in the bile duct and duodenum. Bile from rat 1 was diverted to the duodenum of rat 2, whereas bile from rat 2 was rerouted to the duodenum of rat 1. Next, rat 1 was injected with [^{3}H]cholesterol-loaded macrophages. [^{3}H]Cholesterol secreted via the biliary route was consequently diverted to rat 2 and could thus be quantified from the feces of that rat. On the other hand, [^{3}H]cholesterol tracer in the feces of rat 1 reflected macrophage-derived cholesterol excreted via TICE. Using this setup, we found that 63% of the label secreted with the fecal neutral sterols had travelled via the biliary route, whereas 37% was excreted via TICE.

Conclusions—TICE and biliary cholesterol secretion contribute to macrophage reverse cholesterol transport in rats. The majority of macrophage-derived cholesterol is however excreted via the hepatobiliary route.

Visual Overview—An online visual overview is available for this article. **(*Arterioscler Thromb Vasc Biol*. 2017;37:643-646. DOI: 10.1161/ATVBAHA.116.308558.)**

Key Words: bile ■ cholesterol ■ intestines ■ liver ■ macrophages ■ sterols

Uptake of low-density lipoprotein particles by macrophages in the vessel wall via scavenger receptors[1] and pinocytosis[2] causes the formation of foam cells and thereby gives rise to atherosclerotic cardiovascular disease. Accumulated intracellular cholesterol can be removed via the reverse cholesterol transport (RCT) pathway that mediates efflux of cholesterol from macrophages and its subsequent elimination from the body. Thereby RCT can protect against the development and progression of atherosclerotic plaques.[3] Detailed knowledge about the processes underlying RCT is imperative for successful development of therapeutic intervention strategies. Secretion of cholesterol by the liver into the bile and its subsequent excretion with the feces used to be regarded the sole route to dispose cholesterol from the body, but more recently another pathway has been shown to mediate cholesterol removal directly from the blood in a nonbiliary fashion.[4] Although the exact mechanisms driving the cholesterol flux through the so-called transintestinal cholesterol excretion (TICE) pathway have to date remained incompletely understood, it is now broadly accepted that TICE mediates mobilization of substantial amounts of cholesterol.[5,6] TICE accounts for ≈35% of fecal cholesterol removal in humans.[7] In mice, TICE contributes about 30% to fecal cholesterol excretion under nonstimulated conditions but can be potently stimulated, for example, by LXR (liver X receptor) and FXR (Farnesoid X receptor) agonists, as well as plant sterols.[4,8,9] Liver-derived apolipoprotein (apo) B-containing lipoproteins may deliver cholesterol to the basolateral side of enterocytes for uptake via the low-density lipoprotein receptor and other receptors.[6] After intracellular trafficking to the apical side via poorly defined mechanisms, cholesterol is effluxed into the intestinal lumen via ATP-binding cassette transporters G5 and G8[8] and, conceivably, ABCB1a/b.[10] Whether or not TICE is also involved in macrophage RCT has been fervidly debated. Whereas some studies report that macrophage RCT is almost exclusively mediated via the hepatobiliary route,[11–13] others suggest that it is mainly mediated via TICE.[14] Results reported

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Nonstandard Abbreviations and Acronyms	
FNS	fecal neutral sterols
RCT	reverse cholesterol transport
TICE	transintestinal cholesterol excretion

to date were either obtained in genetic models in which normal hepatic cholesterol fluxes were severely perturbed[11,12,14] or in bile duct ligation models[11,13] lacking phospholipid/bile acid mixed micelles in the intestine that are essential for functional TICE.[15] These deviations from normal physiology conceivably explain the contrasting results, stressing the need for more suitable animal models. In this study, we assessed the respective contributions of biliary cholesterol secretion and TICE to macrophage RCT, under physiological conditions, using an approach in which bile is mutually exchanged between unanesthetized rats for prolonged periods of time.

Materials and Methods

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

Results

To separate cholesterol traffic via the biliary route from TICE, bile flow was mutually exchanged between rats (Figure 1; Methods in the online-only Data Supplement). Groups of 2 weight-matched rats were designated as a pair, and both rats were surgically equipped with cannulas in the bile duct and duodenum.[16] Bile from rat 1 was diverted to the duodenum of rat 2, whereas bile from rat 2 was rerouted to the duodenum of rat 1. Under these circumstances, both rats had an essentially natural delivery of phospholipid/bile acid micelles into the intestine. Next, rat 1 was injected with [^{3}H]cholesterol-loaded macrophage foam cells. [^{3}H]Cholesterol secreted via the biliary route was consequently diverted to rat 2 and could thus be quantified from the feces of this rat. On the other hand, [^{3}H]cholesterol tracer in the feces of rat 1 represented macrophage-derived cholesterol excreted via TICE. To prevent confounding by intestinal cholesterol absorption, ezetimibe was added to the diets. Cholesterol absorption was almost completely blocked on ezetimibe treatment (Figure IA in the online-only Data Supplement).

Body weight and food consumption were stable during the experimental period, indicating that the procedure was well tolerated (Figure IB through ID in the online-only Data Supplement). Serum transaminases were not elevated compared with control rats that did not have surgery, indicating that the procedure did not cause liver damage (Figure IE and IF in the online-only Data Supplement). No differences in feces production or in mass excretion of fecal neutral sterols (FNS) and bile acids were observed (Figure II in the online-only Data Supplement). In keeping with efficient inhibition of intestinal cholesterol absorption, significant amounts of tracer could only be detected in plasma of animals injected with [^{3}H]cholesterol-loaded macrophages (Figure 2A). Because cholesterol is the substrate for bile acid synthesis, macrophage-derived ^{3}H-label can be transferred to those molecules during their synthesis. Bile acids can freely circulate between the rats in this model and therefore transfer radiolabel from one rat to another. As the time needed for enterohepatic cycling of bile acids is only 2 to 3 hours,[16] labeled bile acids are rapidly distributed over both rats of a pair during bile exchange. However, bile acids are very efficiently cleared from the circulation keeping plasma levels very low. Therefore, potential radioactivity in plasma associated with bile acids synthesized from [^{3}H]cholesterol is expected to be minimal. In contrast to plasma, bile acid concentrations in bile are high. Indeed, radioactivity in bile was predominantly because of the incorporation of tritium label into bile acids (data not shown). No overt differences in the appearance of ^{3}H-tracer in bile were observed (Figure 2B), indicating that the bile acid pools mixed well, and bile acids were successfully circulating between the rats. In line with the results obtained from plasma, significant amounts of tracer could only be recovered from livers of the macrophage-injected rats (Figure IIIA in the online-only Data Supplement). Tracer recovery from the FNS fractions reveals the relative contribution of the hepatobiliary pathway and TICE to RCT. Of note, substantial amounts of tracer were detected within the FNS of macrophage-injected and noninjected rats, illustrating that TICE and biliary cholesterol secretion both contribute to macrophage RCT (Figure 2C). Tracer recovery in the neutral sterol fraction of the feces from

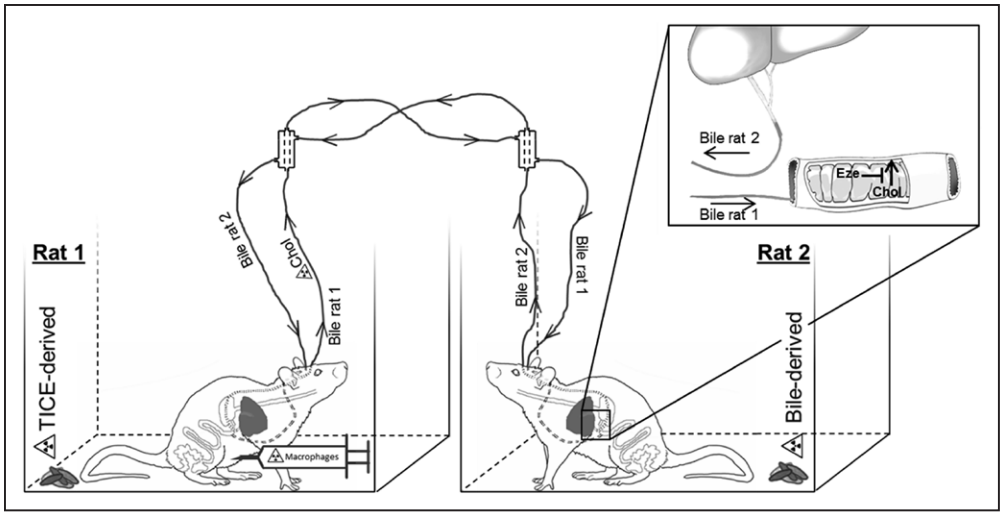

Figure 1. Schematic representation of the continuous bile exchange model in rats that was used to assess the respective contribution of transintestinal cholesterol excretion (TICE) and biliary cholesterol secretion to macrophage reverse cholesterol transport. Chol indicates cholesterol; and Eze, ezetimibe.

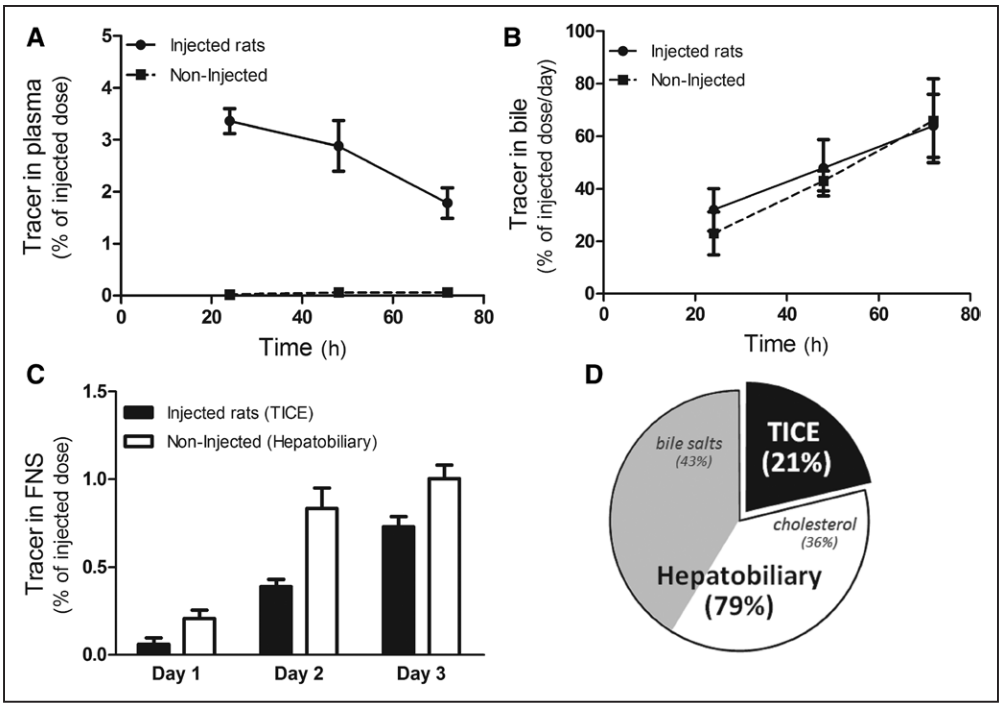

Figure 2. A, Tracer in plasma and (**B**) bile of 4 pairs of rats. Injected rats are the animals that received the [^{3}H]cholesterol-loaded macrophage foam cells, whereas no cells were administered to rats labeled noninjected. **C**, Recovery of tracer within the fecal neutral sterols of the macrophage-injected (rat 1) and noninjected (rat 2) animals, reflecting transintestinal cholesterol excretion (TICE) and hepatobiliary-derived reverse cholesterol transport (RCT), respectively. **D**, Contribution of TICE and hepatobiliary secretion of cholesterol and bile acids to total macrophage reverse cholesterol transport. Data of 4 pairs of rats are plotted, where applicable as mean±SEM. FNS indicates fecal neutral sterols.

the individual pairs and data from 2 additional pairs, included in a second experiment, are shown in Figure IIIB in the online-only Data Supplement. Of the tracer excreted within FNS, 37% was derived from TICE and 63% from biliary cholesterol secretion as reflected by the amounts of tracer present within the FNS fractions of the injected and noninjected rats, respectively. Because tracer excreted within fecal bile acids represents an appreciable fraction of macrophage RCT (Figure IIIC and IIID in the online-only Data Supplement), the actual contribution of TICE to total macrophage RCT is smaller (Figure 2D). Over 3 days, 57% of the total excreted tracer was found within FNS, whereas 43% was present as bile acid. Tracer excretion within FNS of macrophage-injected rats (ie, labeled cholesterol excreted via TICE) accounted for 21% of the total amount of excreted tracer. Because bile acids are committed to the hepatobiliary route, this pathway accounted for 79% of total macrophage RCT (ie, sum of tracer within FNS of the noninjected rat and tracer in fecal bile acids of both rats of a couple).

Discussion

Our study demonstrates that TICE and hepatobiliary cholesterol secretion both contribute to macrophage RCT under physiological conditions. Putting our results into perspective of available literature, Temel et al[14] found no difference in macrophage RCT between liver NPC1L1-transgenic (L-NPC1L1tg) mice and wild-type littermates. Because biliary cholesterol secretion was reduced by >90% in L-NPC1L1tg mice, those results suggest that macrophage RCT does not require a functional hepatobiliary cholesterol secretion pathway, and TICE mediates most of the macrophage RCT. In apparent contrast, using mice with hepatic overexpression of human NPC1L1 on an NPC1L1-deficient background, others reported results arguing against a role for TICE in RCT.[12] However, in both models, physiological hepatic cholesterol fluxes are severely disturbed, generating the possibility that adaptive mechanisms were activated. Therefore, the observations might be a result of the perturbation applied in the animal model rather than reflecting normal physiology. Results from bile duct ligation experiments suggested that only hepatobiliary sterol secretion is contributing to RCT, and that TICE does not play a role in this process.[11,13] However, luminal secretion of cholesterol by enterocytes requires the presence of bile acid/phospholipid micelles in the intestine to facilitate this final step of the TICE pathway.[15] As a result of bile duct ligation, the mixed micelles do not reach the intestine. Therefore, in addition to the hepatobiliary route, TICE is also expected to be inhibited under those conditions, making bile duct ligation an unsuitable model to study the involvement of the TICE pathway in RCT. To circumvent the problems associated with the animal models used to date, we used an alternative approach that does allow quantitative assessment of the contribution of both pathways to (macrophage) RCT under physiological conditions. The only deviation from normal physiology in our model was the use of ezetimibe. We cannot exclude the possibility that the use of ezetimibe affected the results of this study because it can stimulate TICE.[7] The use of ezetimibe was, however, necessary to prevent intestinal reabsorption of labeled cholesterol after its secretion into the bile. Synthesis of bile acids is the major pathway of cholesterol catabolism.[17] Bile acids secreted into the

feces indeed substantially contribute to total macrophage RCT. In our current experiment, 43% of the total amount of label present within feces could be attributed to bile acid synthesis.

In conclusion, using continuous bile exchange between rats, we were able to clarify the differential contribution of TICE and biliary cholesterol secretion to macrophage RCT. We demonstrate that TICE indeed functionally contributes to macrophage RCT, although the majority of this process is mediated via the hepatobiliary route under physiological conditions. It would be of interest to exploit the bile exchange model described in this study to explore whether pharmacological interventions that stimulate cholesterol disposal via TICE impact the differential contribution of this pathway and hepatobiliary cholesterol secretion to macrophage RCT.

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Disclosures

None.

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Highlights

- Although transintestinal cholesterol excretion is broadly accepted to contribute to cholesterol removal from the body, its contribution to reverse cholesterol transport has remained controversial.
- Using a bile exchange model, we were able to separate macrophage reverse cholesterol transport via the hepatobiliary route from reverse cholesterol transport via transintestinal cholesterol excretion while maintaining physiological conditions.
- We demonstrate that transintestinal cholesterol excretion and hepatobiliary cholesterol secretion contribute to macrophage reverse cholesterol transport, but the contribution of the hepatobiliary pathway is dominant under physiological conditions.

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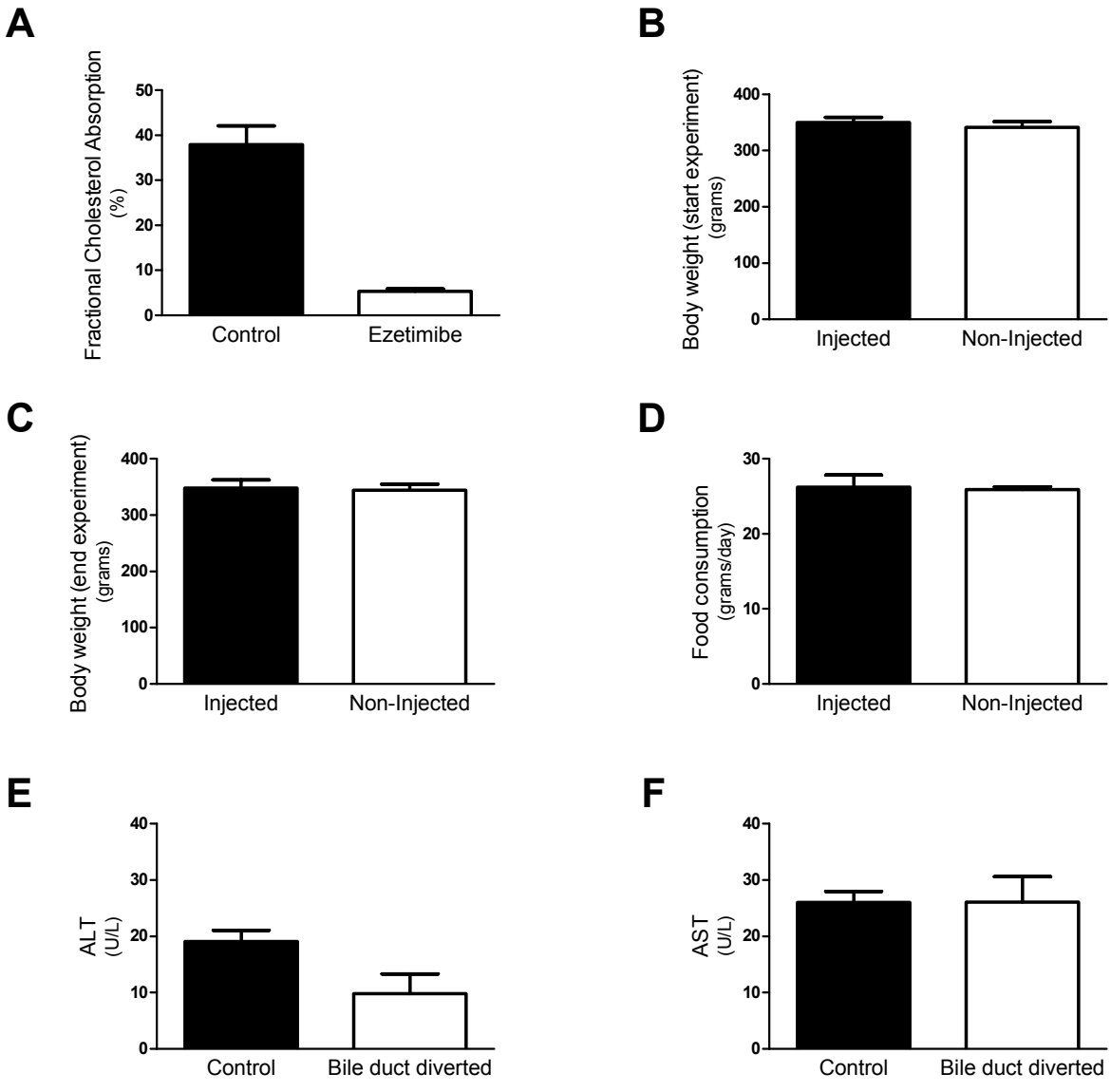

Figure SI. (A) Ezetimibe effectively inhibits cholestrol absorption in rats (n=5 rats/group). (B) Body weights at the start of continuous bile exchange and (C) at the end were stable during the three-day experimental period, indicating that the procedure was well-tolerated (n=4 bile exchange couples). (D) Food intake during the experiment remained stable (n=4 bile exchange couples). (E) Serum levels of alanine aminotransaminase (ALT) and (F) aspartate aminotransaminase (AST) were not elevated in the rats during the bile exchange (n=8) compared to controls that did not undergo surgery (n=5). Rats labeled as 'injected' recieved an intraperitoneal injection with [3H]cholesterol-loaded macrophages. The other rats of the bile exchange couples are labeled as 'non-injected'. Results are expressed as means ± SEM.

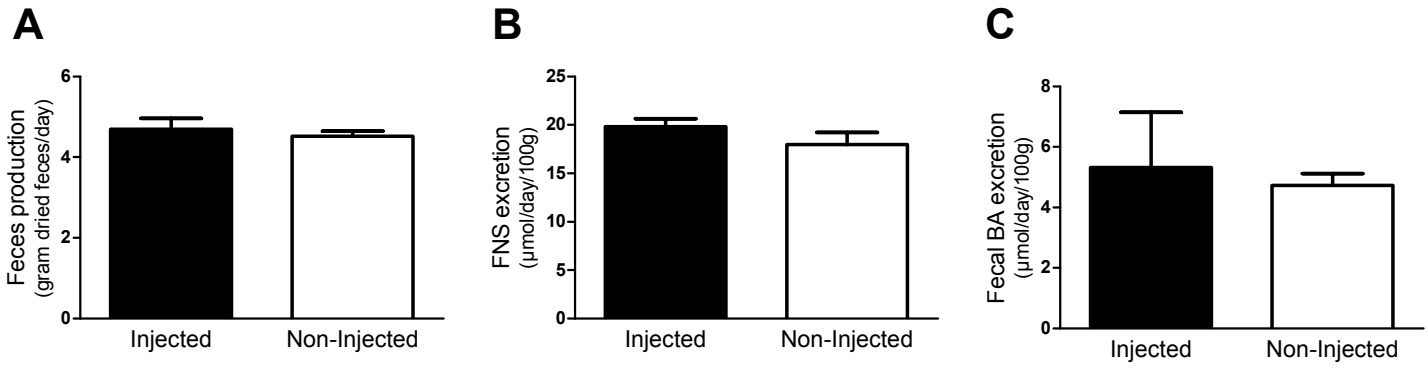

Figure SII. (A) Feces production remained constant during the experiment. (B) No differences in fecal mass excretion of bile acids (BA) and (C) neutral sterols (FNS) were observed between rats that had been injected with the [3H]cholesterol-loaded macrophages ('injected') and the other rats of the bile exchange couples ('non-injected'). Results are expressed as means ± SEM of 4 bile exchange couples.

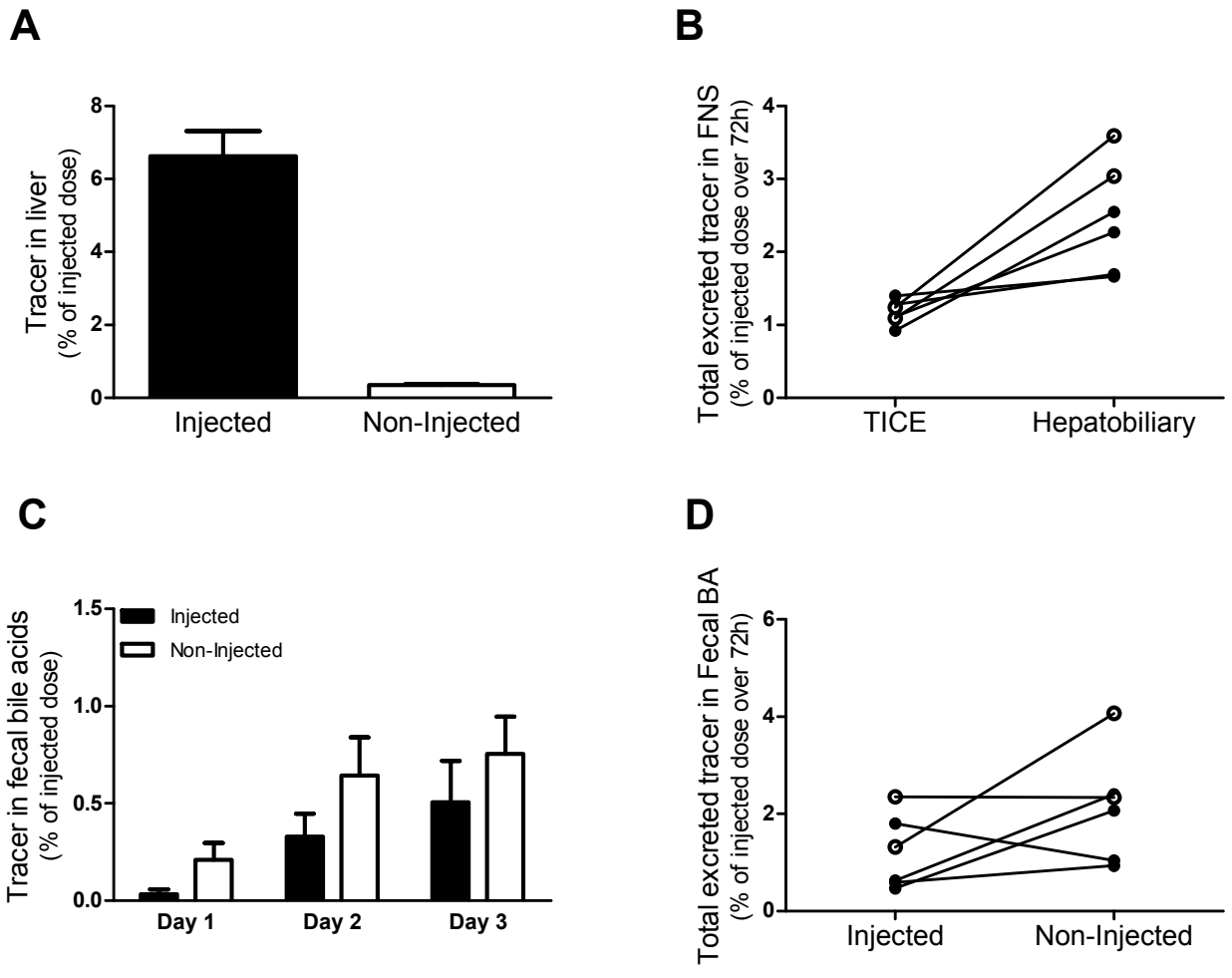

Figure SIII. (A) Recovery of macrophage-derived tracer from the livers of the rats. (B) Total amount of tracer secreted within the fecal neutral sterol (FNS) fraction of the feces of the individual rats during the 3-day period of bile exchange. Data shown with open circles are derived from pairs of rats that were included in a second experiment. Those rats were injected with a different batch of [3H]cholesterol-loaded macrophage foam cells than the rats from the initial experiment (closed circles). (C) Recovery of macrophage-derived tracer from the bile acid fraction of feces on the days after injection of the cells. (D) Total amount of tracer secreted within fecal bile acids of the individual rats during the 3-day period of bile exchange. Data shown with open circles are derived from pairs of rats that were included in a second experiment. Those rats were injected with a different batch of [3H]cholesterol-loaded macrophage foam cells than the rats from the initial experiment (closed circles). 'Injected' rats are the animals that received the [3H]cholesterol-loaded macrophage foam-cells whereas no cells were administered to rats labeled 'non-injected'. Results are given as mean ± SEM of 4 bile exchange couples (A, C) or as paired data of the individual bile exchange couples (B, D), including data from the initial experiment (n=4 couples) as well as data from an additional experiment (n=2 couples) which were injected with a different batch of cholesterol-loaded macrophages.

Supplementary online material

Methods

Animals

Male Wistar rats were obtained from Charles River (Sulzfeld, Germany). The animals were housed in climate controlled animal rooms with alternating 12-hour periods of light and dark. All animals had ad libitum access to water and standard rodent diet (Arie Blok, Woerden, The Netherlands). Three days before the start of the macrophage RCT experiment, the diet was supplemented with 0.005% Ezetimibe (Ezetrol, SP Labo, Heist-op-den-Berg, Belgium). The experiments were performed in conformity with the Dutch law on the welfare of laboratory animals and the experimental protocols were approved by the responsible ethics committee of the University of Groningen.

Cholesterol absorption measurement

Cholesterol absorption was determined essentially as described elsewhere.[1] Rats were injected intravenously with D_5-cholesterol (1.5 mg) and orally gavaged with D_7-cholesterol (3.0 mg). Tracer appearance in the circulation was monitored from blood spots were taken from the tail vein at 0, 3, 6, 12, 24, 48, 72, 92, 120, 144, and 168 hours after administration. Cholesterol absorption was calculated from the ratio of the area under the curve of the oral and intravenously administered tracers, corrected respective doses applied.

Bile duct diversion and bile exchange

Cannulation of the bile duct and duodenum was performed as described previously.[2] Rats were anesthetized with isoflurane, the abdomen was opened and the bile duct was cannulated. Additionally, a cannula was put into the proximal duodenum to serve as bile inflow cannula. Both cannulas were tunneled subcutaneously and exteriorized on top of the head, were they were connected to metal in- and outlets which were fixed on the skull. The cannula coming from the bile duct was connected to the duodenal cannula with a piece of tubing, essentially creating an extended bile duct, to allow the animals to recover for 5-6 days. Buprenorphine (0.0025 mg/kg) was injected subcutaneously at 0, 12 and 24 h after surgery to alleviate discomfort. After recovery, bile exchange was started by connecting the bile cannula of one rat of a designated couple to the duodenal cannula of the other rat and

vice versa. Dual-channel swivels were used to prevent coiling of the tubing. The time needed for bile from one rat to reach the intestine of the other rat, owing to the length of the tubing and dead volume of the swivels, was approximately 20 min. After connecting the biliary circulation of the rats to each other, one rat of each couple was injected with [^{3}H]cholesterol-loaded macrophages and RCT was determined as described below.

Assessment of macrophage reverse cholesterol transport

In vivo macrophage RCT experiments were performed from day 6-9 after bile duct diversion surgery. Macrophages were obtained from male Wistar rats after intraperitoneal injection of 3 ml 4% Brewer thioglycollate medium (Becton Dickinson, Le Point de Claix, France). Macrophages were harvested by peritoneal lavage three days after injection of the thioglycollate. The cells were plated in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, UT, USA) and penicillin (100 U/ml)/streptomycin (100 µg/ml) (Invitrogen). They were allowed to adhere for 4 h at 37 °C in a humidified incubator under 5% CO2. Non-adherent cells were then removed by washing twice with PBS followed by loading of the macrophages with 50 µg/ml acetylated low density lipoprotein (LDL) and 3 µCi/ml [^{3}H]cholesterol (Perkin Elmer, Boston, MA, USA) for 24 h. Next, the macrophages were washed twice with PBS, and equilibrated in RPMI 1640 medium containing penicillin (100 U/ml)/streptomycin (100 µg/ml) and 2% bovine serum albumin (BSA, Sigma) for 18 h. Immediately before injection, cells were lifted using 10mM EDTA in PBS and resuspended in RPMI 1640 medium without additives. One rat of each designated bile exchange couple was intraperitoneally injected with [^{3}H]cholesterol-loaded macrophages (8.7 . 10^6 dpm per rat; approximately 40 million cells). After injection, small blood samples from all rats were obtained by tail bleeding at 24 h intervals for three days. Tracer in plasma was measured by liquid scintillation counting (Packard 1600CA Tri-Carb, Packard, Meriden, CT, USA). Total tracer levels in plasma were calculated based on the estimation that plasma volume in rats equals to 4.1% of body weight.[3] Bile samples were collected every 24 h for three minutes from the tubing between the rats. Because connector parts were placed in the tubing halfway between both rats it could be temporarily disconnected, allowing bile collection without affecting the animals. Bile samples were taken at ground level of the cage and bile collected during the first minute after uncoupling the tubing was discarded to ensure that measured bile flow properly reflected the actual bile flow during the experiment. Tracer in bile was measured as described above for plasma and expressed as percent of injected dose secreted in bile per day. At the end of the experimental period, livers were excised, snap-frozen in liquid nitrogen and stored at -80 °C.

Counts from a respective piece of liver were determined following solubilization of the tissue (Solvable, Packard, Meriden, CT, USA) and were related to total liver mass. Feces was collected in periods of 24 h up to 72 h. Fecal samples were freeze dried, weighed, and thoroughly ground. Neutral sterols were separated from bile acids by first heating 50 mg sample for 2 h at 80 °C in alkaline methanol and then extracting the neutral sterols three times with petroleum ether. Tracer recovered from the neutral sterol and acidic sterol fractions was related to the total amount of feces produced per day. Tracer recovery was expressed as percentage relative to the administered tracer dose.

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