

# Biliary Sterol Secretion Is Not Required for Macrophage Reverse Cholesterol Transport

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## SUMMARY

Recent evidence suggests that the intestine may play a direct facilitative role in reverse cholesterol transport (RCT), independent of hepatobiliary secretion. In order to understand the nonbiliary pathway for RCT, we created both genetic and surgical models of biliary cholesterol insufficiency. To genetically inhibit biliary cholesterol secretion, we generated mice in which Niemann-Pick C1-Like 1 (NPC1L1) was overexpressed in the liver. Compared to controls, NPC1L1<sup>Liver-Tg</sup> mice exhibit a >90% decrease in biliary cholesterol secretion, yet mass fecal sterol loss and macrophage RCT are normal. To surgically inhibit biliary emptying into the intestine, we have established an acute biliary diversion model. Strikingly, macrophage RCT persists in mice surgically lacking the ability to secrete bile into the intestine. Collectively, these studies demonstrate that mass fecal sterol loss and macrophage RCT can proceed in the absence of biliary sterol secretion, challenging the obligate role of bile in RCT.

## INTRODUCTION

Nearly 40 years ago, John Glomset (Glomset, 1968) presented the seminal framework of reverse cholesterol transport (RCT), which was described as a process by which peripheral cholesterol is returned to the liver via high-density lipoproteins (HDLs) for secretion into bile and excretion through the feces. In the context of atherosclerosis, RCT is thought to involve HDL-mediated efflux of cholesterol from the arterial wall, specifically from cholesterol-laden macrophages (Wang and Rader, 2007). In the current view of macrophage RCT, HDL-mediated delivery of peripheral cholesterol to the liver directly promotes biliary and fecal excretion (Lewis and Rader, 2005). Given this model, plasma HDL levels should accurately predict both biliary sterol secretion and fecal sterol loss. However, in mice with extremely low HDL levels, biliary and fecal sterol loss is normal (Xie et al., 2009; Groen et al., 2001; Jolley et al., 1998). In addition, biliary sterol levels do not accurately predict fecal sterol loss in several mouse models of altered hepatic cholesterol metabolism (Brown et al., 2008; Plosch et al., 2002; Yu et al., 2002). Collectively, this has led us to believe that fecal cholesterol loss likely originates

from two distinct excretory routes: (1) the classic hepatobiliary route, and (2) a nonbiliary liver → plasma lipoprotein → small intestine → feces route (Brown et al., 2008). From a therapeutic standpoint, exploiting the nonbiliary route for RCT is a much more attractive option, since excessive augmentation of biliary cholesterol concentrations can promote cholesterol gallstone formation in humans (Cooper, 1991).

Preceding Glomset's (Glomset, 1968) initial model of RCT by 40 years, the existence of a nonbiliary pathway for fecal sterol loss was proposed first by Warren Sperry (Sperry, 1927). Sperry's experiments demonstrated that compared to intact enterohepatic circulation, chronic surgical biliary diversion paradoxically increased fecal neutral sterol loss in dogs. Nearly a half-century later, Pertsemlidis and colleagues confirmed the results of Sperry by demonstrating that biliary diversion resulted in complete loss of fecal acidic sterol output, yet fecal neutral sterol output actually increased ~7-fold (Pertsemlidis et al., 1973). Based on this observation, the authors speculated that "the increased output of fecal neutral steroids could be the result of transfer of plasma cholesterol across the gut wall or due to increased synthesis in the gut." Similar results have been seen under conditions of biliary diversion in rats (Bandsma et al., 1998) and in patients with familial hypercholesterolemia (Deckelbaum et al., 1977). Actually, as early as 1959, it was suggested that nondietary fecal sterol loss in humans consists of two distinct fractions: (1) the traditional fraction coming from hepatobiliary secretion, and (2) an elusive fraction directly secreted by the intestine (Cheng and Stanley, 1959). Unfortunately, many of these early observations have been largely ignored, and the theory that fecal sterol loss derives solely from a biliary origin has become well accepted. However, strong evidence for nonbiliary fecal sterol loss continues to come to light in the age of genetically modified mice.

In a powerful example of nonbiliary fecal sterol loss in mice (Kruit et al., 2005), it was shown that mice lacking the cannicular phospholipid transporter Mdr2, which secondarily have no biliary cholesterol secretion, have normal fecal sterol loss. Furthermore, activation of the liver X receptor (LXR) in *Mdr2*<sup>-/-</sup> mice resulted in large increases in fecal sterol output, supporting the presence of an LXR-inducible nonbiliary pathway for fecal sterol loss (Kruit et al., 2005). Several studies from this same group have subsequently demonstrated the existence of a nuclear hormone receptor-inducible pathway for nonbiliary fecal sterol loss using pioneering approaches such as in situ intestinal perfusion and stable isotope methodologies (van der Veen et al., 2009; Vrins et al., 2009; van der Velde et al., 2007, 2008). However, the relative contribution of biliary and nonbiliary

pathways to the specific process of macrophage RCT is not currently known. Therefore, the purpose of these studies was to determine the direct role of the intestine in macrophage RCT by using novel genetic and surgical mouse models where bile contributes minimally to fecal sterol loss.

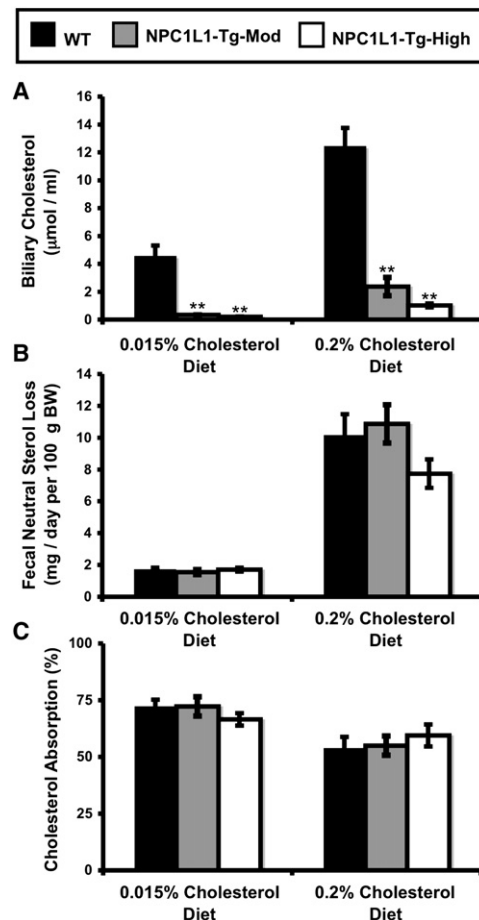
## RESULTS

### NPC1L1<sup>-LiverTg</sup> Mice Serve as an Excellent Model to Study Nonbiliary Fecal Sterol Loss

In order to characterize the role of Niemann-Pick C1-Like 1 (NPC1L1) in hepatobiliary sterol secretion and ezetimibe action, our group previously generated two independent lines of mice transgenically overexpressing NPC1L1 in hepatocytes (Temel et al., 2007). As previously reported, NPC1L1 overexpression in the liver results in a >90% reduction in gall bladder bile cholesterol concentrations (Figure 1A), without affecting biliary phospholipid or bile acid secretion (data not shown; Temel et al., 2007). However, in the face of severely diminished biliary sterol loss, fecal neutral sterol excretion in NPC1L1<sup>-LiverTg</sup> mice is normal (Figure 1B). This disparity indicates that the nonbiliary pathway must completely compensate for biliary sterol insufficiency in NPC1L1<sup>-LiverTg</sup> mice to maintain normal levels of fecal sterol loss. It is important to note that fractional cholesterol absorption (Figure 1C) and the mRNA expression of cholesterol synthetic genes (see Figure S1A available online) in the liver and intestine were normal in NPC1L1<sup>-LiverTg</sup> mice.

### Macrophage RCT Persists in Genetically Modified Mice with Disrupted Biliary Cholesterol Secretion (NPC1L1<sup>-LiverTg</sup>)

In the current view of macrophage RCT, macrophage-derived cholesterol is delivered to the liver by HDL, which shunts this sterol cargo preferentially into the bile for subsequent fecal excretion (Lewis and Rader, 2005; deGoma et al., 2008). We set out to test the specific role of bile in this framework using mice genetically lacking the ability to secrete cholesterol into bile (NPC1L1<sup>-LiverTg</sup>). In these studies, mice were treated with the LXR agonist T0901317, since it is known that LXR activation promotes fecal sterol loss and macrophage RCT (Naik et al., 2006) primarily by augmenting the nonbiliary pathway (Kruit et al., 2005; van der Veen et al., 2009). We have previously seen that NPC1L1<sup>-LiverTg</sup> mice are mildly hypercholesterolemic when fed synthetic diets (Temel et al., 2007). However, when young mice (6–8 weeks old) are maintained on standard rodent chow, NPC1L1<sup>-LiverTg</sup> mice have similar plasma cholesterol levels as those seen in WT littermates (Figure 2A), and the majority of cholesterol is found in HDL (Figure 2B). When treated with the LXR agonist T0901317, both WT and NPC1L1<sup>-LiverTg</sup> mice exhibit mild hypercholesterolemia (164 and 166 mg/dl, respectively), with the majority of the cholesterol elevation seen in large HDL particles (Figure 2B), as has been previously described (Grefhorst et al., 2002). Following [<sup>3</sup>H]-cholesterol-labeled macrophage injection, the plasma [<sup>3</sup>H]-cholesterol recovery was significantly higher in T0901317-treated mice (Figure 2C). As expected, the [<sup>3</sup>H]-cholesterol distribution in plasma (Figure 2D) tracked closely with the cholesterol mass distribution (Figure 2B). Importantly, in WT mice, there was LXR-inducible biliary [<sup>3</sup>H]-cholesterol secretion (Figure 2E),



**Figure 1. Mice Genetically Lacking the Ability to Secrete Cholesterol into Bile Have Normal Fecal Sterol Loss**

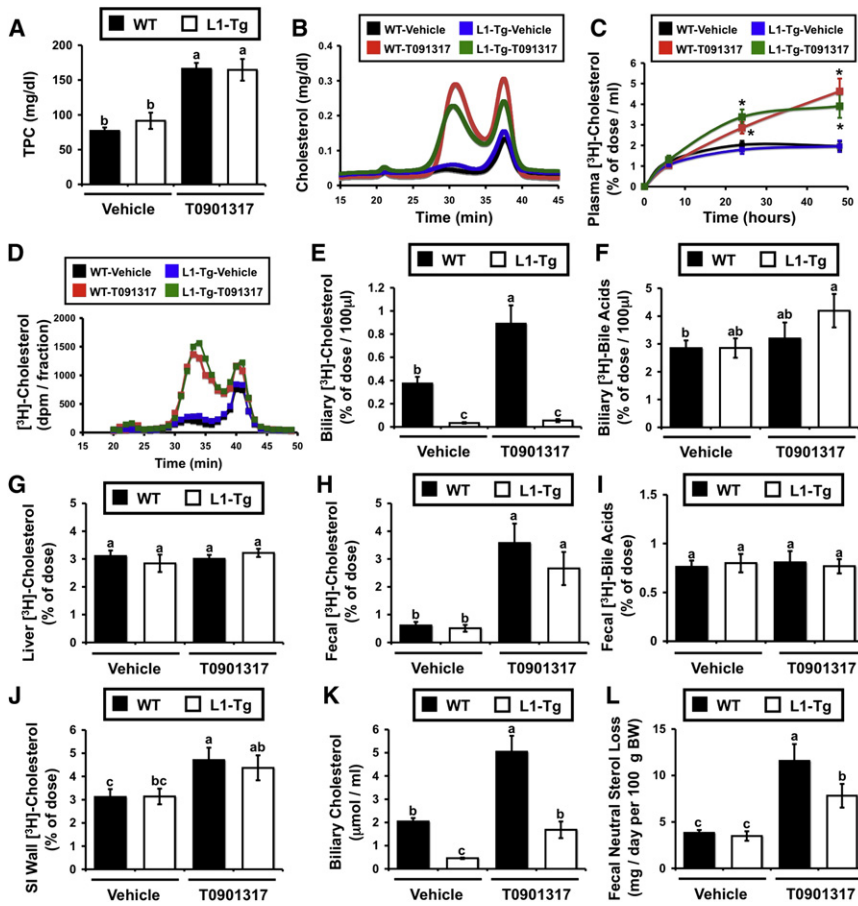
Wild-type mice (WT) or littermates with moderate (NPC1L1-Tg-Mod) or high-level (NPC1L1-Tg-High) overexpression of hepatic NPC1L1 were fed diets containing 0.015% or 0.2% cholesterol (wt/wt) for 6 weeks.

(A) The concentration of cholesterol in gall bladder bile was determined by gas liquid chromatography.

(B) Fecal neutral sterol excretion was determined by gas liquid chromatography.

(C) Fractional cholesterol absorption was determined using the dual fecal isotope method. Data represent the means  $\pm$  SEM from four to seven mice per group, \*\*, significantly different than WT within each diet group,  $p < 0.01$ .

whereas NPC1L1<sup>-LiverTg</sup> mice had barely detectable biliary [<sup>3</sup>H]-cholesterol levels (Figure 2E). However, [<sup>3</sup>H]-bile acid secretion into bile was not different between WT and NPC1L1<sup>-LiverTg</sup> mice in the absence or presence of LXR agonist (Figure 2F), which coincides with mass measurements (Temel et al., 2007; data not shown). However, there was no difference in [<sup>3</sup>H]-cholesterol recovery in the liver between WT and NPC1L1<sup>-LiverTg</sup> mice on either treatment (Figure 2G). In stark contrast to the biliary [<sup>3</sup>H]-cholesterol recovery (Figure 2E), fecal [<sup>3</sup>H]-cholesterol recovery in WT and NPC1L1<sup>-LiverTg</sup> was identical following treatment with vehicle or the LXR agonist (Figure 2H). As previously reported (Naik et al., 2006), LXR activation did increase fecal [<sup>3</sup>H]-cholesterol recovery, but it did so to the same extent in mice with normal enterohepatic circulation (WT) and those



**Figure 2. Macrophage RCT Is Normal in Mice Genetically Engineered to Lack the Ability to Secrete Cholesterol into Bile**

Wild-type mice (■ in bar graphs) or NPC1L1-LiverTg (□ in bar graphs, L1-Tg) littermates were maintained on a standard chow diet in the absence (Vehicle) or presence of the LXR agonist T0901317 (25 mg/kg per day) for 7 days. During the last 48 hr, mice were singly housed, and macrophage to feces RCT was measured as described in the Experimental Procedures.

(A) Total plasma cholesterol (TPC) levels. (B) Mass cholesterol distribution of pooled plasma (n = 4 per pool). (C) Time course of [<sup>3</sup>H]-cholesterol recovery in plasma; \*, significantly different than WT vehicle group within each time point, p < 0.05. (D) [<sup>3</sup>H]-cholesterol distribution of pooled plasma (n = 4 per pool). (E) [<sup>3</sup>H]-cholesterol recovery in newly secreted bile. (F) [<sup>3</sup>H]-bile acid recovery in newly secreted bile. (G) [<sup>3</sup>H]-cholesterol recovery in the liver. (H) [<sup>3</sup>H]-cholesterol recovery in the feces. (I) [<sup>3</sup>H]-bile acids recovery in the feces. (J) [<sup>3</sup>H]-cholesterol recovery in the SI wall. (K) Mass biliary cholesterol concentrations in newly secreted bile. (L) Mass fecal neutral sterol loss. Results are combined from two independent experiments. Data in (A), (C), (E), (F), (G), (H), (I), and (J) represent the means ± SEM from seven to eleven mice per group, and data in (K) and (L) represent means ± SEM from five to nine mice per group. Means not sharing a common superscript differ significantly, p < 0.05.

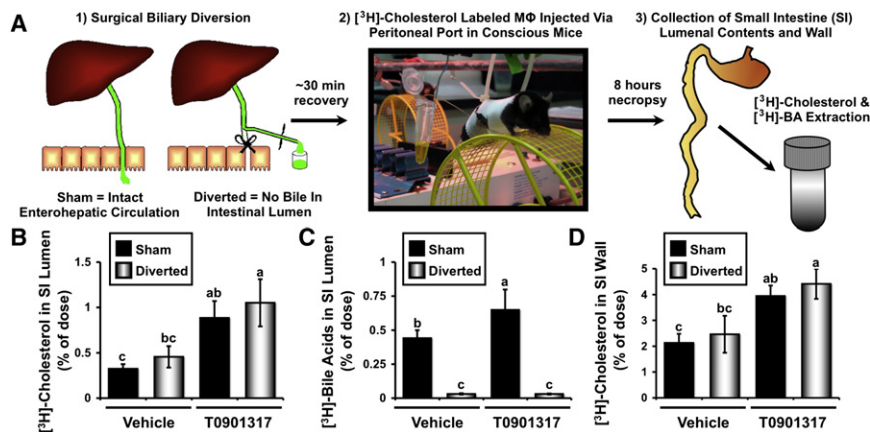
genetically lacking the ability to secrete cholesterol into bile (NPC1L1-LiverTg) (Figure 2H). This effect seems to be specific to neutral sterol loss, since fecal [<sup>3</sup>H]-bile acid recovery was not significantly different between any of the groups (Figure 2I).

Interestingly, the [<sup>3</sup>H]-cholesterol tracer recovery in the small intestinal (SI) wall (Figure 2J) was as high as levels seen in the liver (3%–5% of the dose; Figure 2G). The recovery in the SI wall was not significantly different between WT and NPC1L1-LiverTg mice but was modestly increased by LXR activation (Figure 2J). When examining cholesterol mass, vehicle-treated NPC1L1-LiverTg mice compared to WT mice had a 78% reduction in cholesterol concentration in bile (Figure 2K), yet fecal neutral sterol loss was similar between the two genotypes (Figure 2L). LXR activation did result in significant increases in biliary cholesterol in both genotypes (Figure 2K), but the levels seen in NPC1L1-LiverTg mice only reached that of vehicle-treated WT mice (Figure 2K). In contrast, fecal neutral sterol loss in T0901317-treated WT and NPC1L1-LiverTg mice was significantly higher than that of vehicle-treated counterparts (Figure 2L), further supporting the disconnect between biliary sterol secretion and fecal neutral sterol loss in these mice.

### Surgical Biliary Diversion Reveals the Existence of a Nonbiliary Pathway for Macrophage RCT

Although NPC1L1-LiverTg mice serve as an excellent model where nonbiliary sterol loss predominates, there is a small residual

amount of biliary sterol loss in this genetic model (Figures 1A and 2K). In order to more definitively test whether bile is required for macrophage RCT, we set out to create a surgical model where there would be no biliary emptying into the small intestine, without obstructing bile flow. To do this, we surgically diverted bile flow away from the small intestine (Figure 3A). Once this biliary diversion, or an appropriate sham surgery, was complete, mice were allowed to recover from anesthesia and received a [<sup>3</sup>H]-cholesterol-labeled macrophage foam cell dose via an externalized port in order to avoid accidental injection directly into the intestine. Quite strikingly, the appearance of [<sup>3</sup>H]-cholesterol in the intestinal lumen was no different between sham-operated and surgically diverted mice (Figure 3B). Furthermore, LXR activation resulted in significant increases in the luminal appearance of [<sup>3</sup>H]-cholesterol in both sham and diverted animals (Figure 3B), further supporting the notion that LXR activation promotes primarily the nonbiliary pathway for fecal sterol loss (Kruit et al., 2005; van der Veen et al., 2009). Importantly, the luminal appearance of [<sup>3</sup>H]-bile acids was completely blocked by surgical biliary diversion (Figure 3C), which agrees with previous mass data in dogs (Sperry, 1927; Pertsemilidis et al., 1973). These data support the notion that bile is the only route for hepatic bile acids to reach the SI lumen, while cholesterol delivery into the intestine and feces can persist in the absence of biliary return (Sperry, 1927; Pertsemilidis et al., 1973; Dietschy, 1968; Figure 3). Interestingly, at this early 8 hr time point after



### Figure 3. Surgical Biliary Diversion Reveals the Existence of a Nonbiliary Pathway for Macrophage RCT

Wild-type C57BL/6N mice were maintained on a standard chow diet in the absence (Vehicle) or presence of the LXR agonist T0901317 (25 mg/kg per day) for 7 days.

(A) The experimental design is as follows: following 7 days of vehicle or LXR treatment, mice were either sham operated or underwent complete surgical bile diversion as described in the **Experimental Procedures**. Thereafter, mice received a [<sup>3</sup>H]-cholesterol-labeled macrophage (MΦ) dose via an externalized peritoneal port in order to ensure no dose was accidentally injected into the intestine. After 8 hr, the SI luminal contents and wall were collected and extracted to separate [<sup>3</sup>H]-cholesterol and [<sup>3</sup>H]-bile acids.

(B) [<sup>3</sup>H]-cholesterol recovered in the SI luminal contents.

(C) [<sup>3</sup>H]-bile acids recovered in the SI luminal contents.

(D) [<sup>3</sup>H]-cholesterol recovered in the SI wall. Data represent the means ± SEM from five to nine mice per group, and means not sharing a common superscript differ significantly,  $p < 0.05$ .

[<sup>3</sup>H]-cholesterol-labeled macrophage injection, 2%–4% of the dose can be found in the SI wall (Figure 3D). There was no difference in the SI wall recovery between sham and diverted animals, yet LXR activation did significantly increase [<sup>3</sup>H]-cholesterol recovery in the SI wall under conditions of intact and diverted enterohepatic circulation (Figure 3D). Collectively, these data support the notion that macrophage RCT can persist in the complete absence of biliary contributions.

## DISCUSSION

Although it is generally accepted that macrophage RCT depends entirely on the ability of the liver to secrete HDL-delivered cholesterol into bile (Lewis and Rader, 2005), results from this study suggest that the current conceptual framework of macrophage RCT requires significant modification. The major findings of the current study are the following: (1) mice genetically lacking the ability to adequately secrete cholesterol into bile (NPC1L1<sup>-LiverTg</sup> mice) have normal LXR-inducible macrophage RCT, and (2) mice surgically lacking biliary contributions to the intestine have normal LXR-inducible macrophage RCT. Importantly, the genetic and surgical models defined in this work should continue to serve as useful tools to further interrogate the molecular mechanisms mediating nonbiliary fecal sterol loss and macrophage RCT.

The relative contribution of biliary versus nonbiliary pathways to fecal sterol loss has not been fully defined. Using an intestinal perfusion system, it was estimated that ~44% of total fecal sterol output originated from nonbiliary sources in humans (Simmonds et al., 1967). In normal chow-fed C57BL/6J mice, the nonbiliary route accounts for 33% (76 μmol/kg/day) of total fecal neutral sterol loss (van der Veen et al., 2009) and roughly 20% of total fecal sterol loss in FVB mice (Kruit et al., 2005). However, it is important to point out that nonbiliary fecal sterol loss is quite sensitive to pharmacological manipulation. To this end, LXR

activation can dramatically augment nonbiliary macrophage RCT (Figures 2 and 3) and increase mass fecal neutral sterol loss (Kruit et al., 2005; van der Veen et al., 2009). In fact, LXR activation in C57BL/6J increases the contribution of nonbiliary fecal sterol loss from ~33% of the total (76 μmol/kg/day) in vehicle-treated mice to ~63% of the total (442 μmol/kg/day) in T0901317-treated mice (van der Veen et al., 2009). Furthermore, activation of the peroxisome proliferator-activated receptor δ (PPAR-δ) promotes nonbiliary fecal sterol loss in mice (Vrins et al., 2009). Importantly, now in three independent genetically modified mouse models (ABCG5/G8<sup>-/-</sup>, Mdr2<sup>-/-</sup>, and NPC1L1<sup>-LiverTg</sup>) that have severely diminished biliary cholesterol secretion, fecal sterol loss is only modestly decreased (Yu et al., 2002) or not altered at all (Kruit et al., 2005; Figure 1). This clearly indicates that the nonbiliary pathway must be able to adequately compensate for biliary insufficiency to maintain normal fecal sterol loss in rodents. Collectively, these data support the idea that fecal sterol loss is a mixture of dietary, biliary, and intestinally derived sterols, and the origins of the latter source likely originate from the plasma compartment (Brown et al., 2008; Kruit et al., 2005; van der Veen et al., 2009). Given the plasma source of intestinally derived fecal sterols, and the central role of the liver in lipoprotein metabolism, one must consider the liver as a potential site of organization for nonbiliary fecal sterol loss.

The classic view of RCT involves the delivery of peripheral cholesterol via HDL to the liver for secretion into bile (Lewis and Rader, 2005; deGoma et al., 2008). In parallel, we believe that the liver also plays a gatekeeper role for nonbiliary fecal sterol loss by repackaging peripheral cholesterol into nascent plasma lipoproteins that are destined for subsequent intestinal delivery (Brown et al., 2008). Probably not coincidentally, all of the mouse models described in which nonbiliary fecal sterol loss is apparent (Figure 1; Brown et al., 2008; Kruit et al., 2005; Temel et al., 2007) represent conditions where free cholesterol

could potentially accumulate in the liver due to defects in normal elimination pathways. It remains possible that under conditions where hepatic free cholesterol burden becomes too excessive for disposal through esterification or biliary secretion (i.e., ACAT2 ASO treatment [Brown et al., 2008], NPC1L1<sup>-Liver-Tg</sup> mice [Figure 1; Temel et al., 2007], or *Mdr2*<sup>-/-</sup> mice [Kruit et al., 2005]), an alternative plasma-based route for direct intestinal secretion and fecal disposal is utilized. In previous work, we were able to show that the liver can secrete lipoprotein particles that preferentially deliver cholesterol to the proximal small intestine for fecal excretion (Brown et al., 2008). However, whether these liver-derived lipoproteins represent nascent VLDL particles, nascent HDL particles, or some novel lipoprotein remains to be addressed. Interestingly, unlike the genetic models of biliary cholesterol insufficiency (*ABCG5/G8*<sup>-/-</sup>, *Mdr2*<sup>-/-</sup>, and NPC1L1<sup>-LiverTg</sup>), acute surgical diversion of bile is a situation in which normal elimination pathways such as esterification or biliary secretion are not obviously impaired, yet de novo cholesterol synthesis is augmented (Figure S1; Dietschy, 1968; Deckelbaum et al., 1977; Pertsemididis et al., 1973; Bandsma et al., 1998). In this case it has been assumed that the origin of fecal sterols in bile-diverted animals comes from the enhanced de novo synthesis (Dietschy, 1968; Deckelbaum et al., 1977; Pertsemididis et al., 1973; Bandsma et al., 1998). However, our data suggest that a small portion of the fecal sterols present in bile-diverted mice is derived from direct intestinal secretion of macrophage-derived cholesterol. This is supported by the fact that we have added an intact [<sup>3</sup>H]-cholesterol molecule delivered via J774 macrophages, excluding any contribution from endogenous synthesis.

Although macrophage RCT is agreed to be key to the regression of atherosclerosis, it is important to discuss the quantitative importance of macrophages in RCT and centripetal flux of cholesterol into the feces. To put macrophage RCT in perspective to the total process of centripetal cholesterol flux, it has been estimated that there are only  $\sim 1 \times 10^8$  macrophages in the adult mouse (Lee et al., 1985). Hence, the number of macrophages in the whole body represents only a small fraction of the other cell types present, making the relative contribution of macrophages to mass fecal neutral sterol loss extremely small. Even though macrophages are a minor contributor to mass fecal neutral sterol loss, macrophage-specific RCT is likely highly relevant to atherosclerosis regression in the artery wall (Lewis and Rader, 2005). Therefore, it is also important to discuss whether the macrophage RCT assay used in this work truly recapitulates the process thought to occur in the artery wall. Since the original description (Zhang et al., 2003) of the assay used in our studies, there have been over 30 independent studies described in the literature utilizing this method. Collectively, these studies indicate that once the [<sup>3</sup>H]-cholesterol-labeled J774 macrophages are injected, these cells remain primarily within the peritoneal cavity, and the appearance of [<sup>3</sup>H]-cholesterol in the plasma and tissues is not likely due to the direct transport of the tracer by intact J774 cells (Zhang et al., 2003; Naik et al., 2006; Wang et al., 2007; Rader et al., 2009). Instead, the bulk of evidence suggests that a small fraction of [<sup>3</sup>H]-cholesterol tracer is removed from the J774 macrophages by traditional efflux mechanisms, and once effluxed this tracer is metabolized in a manner comparable to endogenous cholesterol mass

(Zhang et al., 2003; Naik et al., 2006; Wang et al., 2007; Rader et al., 2009). It is important to note that the seemingly modest fecal recovery (1%–4%) of injected [<sup>3</sup>H]-cholesterol (Figure 2H) may actually be substantial, given that only 1%–5% was found in the plasma over the 48 hr period (Figure 2C). Whether this assay truly recapitulates the process of macrophage RCT from the artery wall is still a matter of debate, but there is good evidence that determining the rate of macrophage RCT using this assay accurately predicts atherosclerosis burden in mice (Rader et al., 2009). However, given the assay limitations, and the relatively small contributions of macrophages to fecal sterol loss, it remains critically important for investigators to quantify mass fecal sterol loss to more accurately determine whole-body centripetal cholesterol flux in future studies.

It has been over 80 years since Warren Sperry speculated that a nonbiliary route for fecal neutral sterol loss must exist in dogs (Sperry, 1927). Unfortunately, this alternative pathway has been largely ignored, and very little progress has been made to identify the molecular mechanisms that define it. There is now strong evidence in mice (Figures 1–3; Kruit et al., 2005; van der Velde et al., 2007, 2008; van der Veen et al., 2009; Vrins et al., 2009; Brown et al., 2008; Plosch et al., 2002), and mounting evidence in man (Simmonds et al., 1967; Cheng and Stanley, 1959; Deckelbaum et al., 1977), that a nonbiliary pathway for fecal sterol loss indeed exists. In order to more fully understand this pathway, the following challenges will need to be addressed: (1) establishing quantitative methods to measure nonbiliary fecal sterol loss in primates and man, (2) identifying the intestinal transport proteins/receptors involved, (3) characterizing the lipoprotein metabolism that is requisite for nonbiliary fecal sterol loss, and (4) identifying bona fide drug targets to specifically modulate the intestinal component of RCT. Advancement in these areas has the potential to open new therapeutic opportunities targeting the intestine as an inducible sterol excretory organ.

## EXPERIMENTAL PROCEDURES

### In Vivo Macrophage RCT Studies in NPC1L1<sup>-LiverTg</sup> Mice

In vivo measurement of macrophage RCT was conducted essentially as described by Rader and colleagues (Zhang et al., 2003; Naik et al., 2006), with minor modifications. Extensive descriptions of the mouse models used, cell culture protocols, and RCT method are included in the Supplemental Information.

### In Vivo Macrophage RCT Studies with Acute Surgical Biliary Diversion

C57BL/6N mice maintained on standard rodent chow were gavaged with either vehicle or 25 mg/kg T0901317 daily for 7 consecutive days prior to surgical biliary diversion. The surgical procedure used to acutely divert biliary emptying into the intestine and macrophage RCT protocol is included in the Supplemental Information.

### Plasma Lipoprotein Distribution Analysis

Detailed description of plasma lipid analyses is included in the Supplemental Information.

### Quantitative Real-Time PCR

RNA extraction and quantitative real-time PCR (qPCR) were conducted as previously described (Brown et al., 2008) using the  $\Delta\Delta$ -CT method. Primers used for qPCR are available upon request.

### Statistical Analysis

Data are expressed as the mean  $\pm$  standard error of the mean (SEM). All data were analyzed using two-way analysis of variance (ANOVA) followed by Student's *t* tests for post hoc analysis. Differences were considered significant at  $p < 0.05$ . All analyses were performed using JMP version 5.0.12 (SAS Institute; Cary, NC) software.

### SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and one figure and can be found with this article online at [doi:10.1016/j.cmet.2010.05.011](https://doi.org/10.1016/j.cmet.2010.05.011).

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