Liver X receptor-mediated activation of reverse cholesterol transport from macrophages to feces in vivo requires ABCG5/G8

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Abstract Liver X receptor (LXR) agonists increase both total fecal sterol excretion and macrophage-specific reverse cholesterol transport (RCT) in vivo. In this study, we assessed the effects of ABCG5/G8 deficiency as well as those of LXR agonist-induction of RCT from macrophages to feces in vivo. A [3H]cholesterol-labeled macrophage cell line was injected intraperitoneally into ABCG5/G8-deficient (G5/G8$^{-/-}$), heterozygous (G5G8$^{+/-}$), and wild-type G5/G8$^{+/+}$ mice. G5/G8$^{-/-}$ mice presented increased radiolabeled HDL-bound [3H]cholesterol 24 h after the label injection. However, the magnitude of macrophage-derived [3H]cholesterol in liver and feces did not differ between groups. A separate experiment was conducted in G5G8$^{+/+}$ and G5G8$^{-/-}$ mice treated with or without the LXR agonist T0901317. Treatment with T0901317 increased liver ABCG5/G8 expression, which was associated with a 2-fold increase in macrophage-derived [3H]cholesterol in feces of G5/G8$^{+/+}$ mice. However, T0901317 treatment had no effect on fecal [3H]cholesterol excretion in G5G8$^{-/-}$ mice. Additionally, LXR activation stimulated the fecal excretion of labeled cholesterol after an intravenous injection of HDL-[3H]cholesteryl oleate in G5/G8$^{+/+}$ mice, but failed to enhance fecal [3H]cholesterol in G5/G8$^{-/-}$ mice. ▪ Our data provide direct in vivo evidence of the crucial role of ABCG5 and ABCG8 in LXR-mediated induction of macrophage-specific RCT.—Calpe-Berdiel, L., N. Rotllan, C. Fiévet, R. Roig, F. Blanco-Vaca, and J. C. Escolà-Gil. **Liver X receptor-mediated activation of reverse cholesterol transport from macrophages to feces in vivo requires ABCG5/G8.** *J. Lipid Res.* 2008. **49:** **1904–1911.**

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The classic reverse cholesterol transport (RCT) pathway involves cholesterol efflux from peripheral tissues to HDL, which is taken up by the liver and finally excreted into bile and feces. Liver X receptor (LXR) α is a nuclear receptor activated by oxysterols (1) that upregulates expression of a number of target genes critical for RCT, including ABCA1, ABCG1, ABCG5, ABCG8, and murine cytochrome P450 family 7 subfamily A polypeptide 1 (CYP7A1) (2–6). LXRα is highly expressed in the liver and at lower levels in the adrenal glands, intestine, adipose tissue, macrophages, lung, and kidney, whereas LXRβ is ubiquitously expressed (7). ABCG5 and ABCG8 heterodimerize into a functional complex (ABCG5/G8) that is crucial for hepatobiliary and intestinal sterol excretion. Genetic deficiency of ABCG5/G8 in humans causes sitosterolemia (8, 9), which is characterized by plant sterol accumulation in plasma and tissues due to increased intestinal absorption and decreased biliary secretion of sterols (10, 11).

It is difficult at present to quantify RCT in humans (12, 13). A new approach was developed to measure RCT from labeled cholesterol incorporated into macrophages and injected into mice liver and feces (14). Follow-up studies demonstrated that the rate of RCT from macrophages to feces may not always be inferred from studies of total RCT (14–17). This is an important point in the context of current efforts to better understand HDL antiatherogenic functions as well as to develop a new test for predicting those functions. Analysis of ABCG5/G8 function in RCT is, there-

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fore, of interest. Overexpression of ABCG5/G8 in transgenic mice and pharmacological activation of LXR result in increased hepatobiliary excretion of cholesterol and increased fecal neutral sterol excretion (18–22), and this action is dependent on ABCG5/G8 (19). Inactivation of both genes simultaneously has the opposite effects on sterol hepatobiliary excretion (23). Importantly, overexpression of both human ABCG5 and -G8 in transgenic mice also reduces intestinal cholesterol absorption (18), although the opposite effect was not observed in ABCG5/G8 double-knockout mice (19, 23, 24). Synthetic LXR ligands stimulate cholesterol efflux from cultured macrophages (6, 25), inhibit atherosclerosis progression, and even promote atherosclerosis regression in mice (26–28). This effect has been shown to depend on the presence of macrophage LXR (28). On the other hand, the synthetic LXR agonist GW3965 promotes specific RCT from macrophages to feces in vivo despite having little effect on HDL cholesterol levels (29). Considering that ABCG5/G8 is not expressed in macrophages, the results of these studies support the concept that LXR activation may modulate this macrophage-specific RCT pathway by increasing biliary cholesterol excretion, presumably through upregulation of ABCG5 and ABCG8 mRNA expression (19). However, the contribution of ABCG5/G8 heterodimer to RCT from macrophages to feces in vivo remains to be directly established (12, 30).

In the present study, we investigated the effects of ABCG5/G8 deficiency as well as those of LXR agonist induction of RCT from a macrophage cell line to feces in vivo.

MATERIALS AND METHODS

Mice and diet

ABCG5 and ABCG8 heterozygous mice (G5G8$^{+/-}$) in the 129S6SvEv $\times$ C57BL/6J background were obtained from Jackson Laboratories (#004670; Bar Harbor, ME) and were crossed to produce wild-type G5G8$^{+/+}$, G5G8$^{+/-}$, and G5G8$^{-/-}$ mice (23, 24). Genotype of the offspring was confirmed by polymerase chain reaction (PCR) using the wild-type and the targeted allele-specific primers recommended by Jackson Laboratories (http://jaxmice.jax.org/pub-cgi/protocols/protocols.sh). For this study, we used 3 to 4 month-old G5G8$^{+/+}$, G5G8$^{+/-}$, and G5G8$^{-/-}$ mice fed a regular chow diet (Rodent Toxicology Diet; B and K Universal, UK) containing 0.02% cholesterol. Male and female mice were used in equal proportions. All animal manipulations began at noon with the mice fed ad libitum. All animal procedures were in accordance with published recommendations of the Guide for the Care and Use of Laboratory Animals (31) and approved by the Institutional Animal Care Committee of the Hospital de la Santa Creu i Sant Pau.

For experiments on LXR-mediated induction of RCT from macrophages to feces in vivo, G5G8$^{+/+}$ and G5G8$^{-/-}$ mice were given one daily oral gavage of vehicle (1.0% v/v methyl sufoxide and 1.0% w/v carboxymethylcellullose medium viscosity) or a dose of 10 mg/kg body weight of LXR agonist T0901317 (Cayman Chemicals, Ann Arbor, MI) dissolved in the vehicle solution at 4 PM, for 5 consecutive days. Mice continued to receive vehicle or drug during the 48 h of the RCT study (2 additional consecutive days). Dose and duration of T0901317 treatment were based on previous studies (19, 32, 33). HDL catabolism and gene expression analyses were studied in independent experiments with G5G8$^{+/+}$ and G5G8$^{-/-}$ mice treated for 7 days with or without T0901317 as described above (n = 6 and n = 5 per group, respectively). At the end of the gene expression experiment, mice were exsanguinated, and two 100 mg portions of the liver were removed and snap-frozen for lipid extraction and quantitative real-time RT-PCR analyses, respectively. Small intestines were also extracted and flushed with ice-cold saline solution for quantitative real-time RT-PCR analyses (34).

Lipid analyses of plasma, liver, and stools

Plasma, HDL, and liver sterol composition (cholesterol, campesterol, and β-sitosterol) was analyzed by GC-MS after lipid extraction with isopropyl alcohol-hexane (2:3, v/v) (34). Plasma levels of murine apolipoprotein A-I (apoA-I) and apoA-II were measured by immunonephelometric assay using specific mouse polyclonal antibodies (35). Stools from individually housed mice collected over 3 days were dried, weighed, and ground to a fine powder, and fecal sterol composition was analyzed by GC-MS after lipid extraction (34). Bile acids of 1 g of feces were extracted in ethanol and used to determine total bile acid content by the 3α-hydroxysteroid dehydrogenase method (Sigma Diagnostics, St. Louis, MO) (34).

TABLE 1. Plasma sterol and apolipoprotein A levels in ABCG5/G8$^{+/+}$ and ABCG5/G8$^{-/-}$ mice fed a chow diet treated with (+) or without (−) LXR agonist T0901317 for 7 days

	ABCG5G8$^{+/+}$		ABCG5G8$^{-/-}$	
	− T0901317	+ T0901317	− T0901317	+ T0901317
Cholesterol (mM)	1.66 ± 0.11	1.94 ± 0.27	1.28 ± 0.11	1.57 ± 0.20
Campesterol (mM)	Nd	Nd	0.48 ± 0.04	0.67 ± 0.09
Sitosterol (mM)	Nd	Nd	1.33 ± 0.11	1.72 ± 0.22
HDL cholesterol (mM)	1.22 ± 0.11	1.87 ± 0.37^a	0.97 ± 0.11	1.44 ± 0.18
HDL campesterol (mM)	Nd	Nd	0.42 ± 0.05	0.49 ± 0.06
HDL sitosterol (mM)	Nd	Nd	0.99 ± 0.11	1.31 ± 0.16
ApoA-I (%)	100.0 ± 7.6	117.6 ± 13.5	120.9 ± 16.4	147.8 ± 4.5
ApoA-II (%)	100.0 ± 11.5	114 ± 17.4	116.4 ± 16.8	148.8 ± 5.5

apoA-I, apolipoprotein A-I; LXR, liver X receptor; Nd, nondetected. Plasma cholesterol, sitosterol, and campesterol levels were analyzed by GC-MS. HDL was isolated from plasma of mice by ultracentrifugation, and cholesterol, sitosterol, and campesterol were determined. Plasma murine apoA-I and apoA-II levels are reported as mean percent change from ABCG5/G8$^{+/+}$ mice given vehicle. Results are expressed as mean ± SEM of five individual animals per group. Factors for converting mM into mg/dl for cholesterol, campesterol, and sitosterol are 38.7, 40.1, and 41.5, respectively.
^{a} P < 0.05 between T0901317-treated and vehicle-treated mice of each genotype.

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Determination of RCT from a macrophage cell line to feces in vivo

P388D1 mouse macrophages (American Type Culture Collection, Manassas, VA) were cultured in 75 cm tissue culture plaques at 5 million cells per plaque and grown to 90% confluence in RPMI 1640 supplemented with 10% FBS (15). P388D1 mouse macrophages were incubated for 48 h in the presence of 5 μCi/ml of [1α,2α(n)-^{3}H]cholesterol (specific activity of 44 × 10^3 cpm/pmol; Amersham Biosciences Europe GmbH, Germany), 100 μg/ml of acetylated LDL, and 10% lipoprotein-depleted serum. These cells were washed, equilibrated, detached with cell scrapers, resuspended in 0.9% (w/v) saline, and pooled before being intraperitoneally injected into mice (15, 16). Cell count and viability were measured by acridine orange and ethidium bromide staining; cell viability was 61 ± 2% (n = 4 independent determinations). In the first experiment, 13 G5G8$^{+/+}$, 9 G5G8$^{+/-}$, and 12 G5G8$^{-/-}$ mice were injected intraperitoneally with [^{3}H]cholesterol-labeled P388D1 mouse macrophages (9.2 × 10^6 cells containing 8.8 × 10^5 cpm in 0.5 ml of saline in each mouse). An additional macrophage RCT experiment was performed in G5G8$^{+/+}$ and G5G8$^{-/-}$ mice treated with or without T0901317 (seven mice per group). In both experiments, mice were individually housed in metabolic cages, and stools were collected over the next two days. Mice were exsanguinated by cardiac puncture at 48 h, and livers were removed after extensive perfusion with saline. Plasma counts per minute was determined at 24 and 48 h by liquid scintillation counting. HDL-associated [^{3}H]cholesterol and non-HDL-associated [^{3}H]cholesterol were measured after precipitation with phosphotungstic acid and magnesium chloride (Roche Diagnostics GmbH, Germany) (15, 16). Liver and fecal lipids were extracted with isopropyl alcohol-hexane and the distribution of [^{3}H]cholesterol between free cholesterol and cholesteryl ester was determined by TLC (15, 16). The [^{3}H]tracer detected in fecal bile acids was determined as previously described (15, 16). The amount of [^{3}H]tracer was also expressed as a fraction of the injected dose.

Metabolism of [^{3}H]cholesteryl oleate HDL

Autologous [^{3}H]cholesteryl oleate-labeled HDL (500,000 cpm; specific activity of 45 × 10^3 cpm/pmol; Amersham Biosciences Europe GmbH) was prepared and injected intravenously into each mouse as described (36). In all cases, [^{3}H]HDL was <5% of the total plasma cholesterol mass (36). Blood was collected in heparinized tubes at the indicated times, and the radioactivity contained in 50 μl of plasma aliquots was determined. This analysis was used to fit an exponential curve to each set of plasma decay data (36). At the end of the experiment, fecal [^{3}H]cholesterol and the [^{3}H]tracer detected in fecal bile acids were determined as described above.

Quantitative real-time RT-PCR analyses

The liver and small intestine of five animals in each experimental group were removed. The entire small intestine was cut into three segments with length ratios of 1:3:2 (duodenum-jejunum-ileum). From the middle of each intestinal segment, 1.5 cm of the duodenal, jejunal, and ileal tissues were cut out and pooled (37). Liver and small intestine RNA were isolated using the trizol RNA isolation method (Gibco/BRL, Grand Island, NY). Total RNA samples were repurified, checked for integrity by agarose gel electrophoresis, and reverse-transcribed with Oligo(dT)$_{15}$ using an Moloney Murine Leukemia virus reverse transcriptase, RNase H$^-$ point mutant to generate cDNA (34). Predesigned validated primers (Assays-on-Demand; Applied Biosystems, Foster City, CA) were used with Taqman probes (38). PCR assays were performed on an Applied Biosystems

Prism 7000 sequence detection system (Applied Biosystems) as described (37). All analyses were performed in duplicate, and relative RNA levels were determined using PGK1 as internal control.
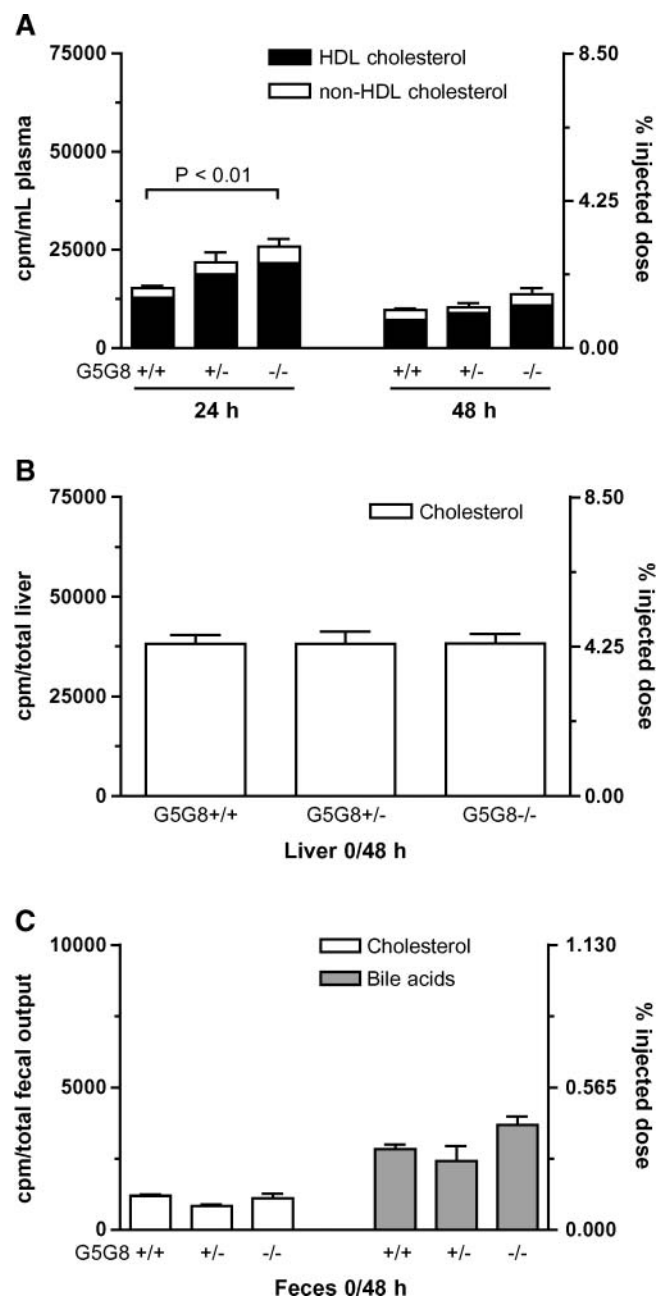

Fig. 1. Reverse cholesterol transport from macrophages to feces in wild-type (G5G8$^{+/+}$), ABCG5/G8 heterozygous (G5G8$^{+/-}$), and ABCG5/G8-deficient (G5G8$^{-/-}$) mice maintained on a regular chow diet. Thirteen G5G8$^{+/+}$ (eight males and five females), nine G5G8$^{+/-}$ (five males and four females), and twelve G5G8$^{-/-}$ (seven males and five females) individually housed mice were injected intraperitoneally with [3H]cholesterol-labeled P388D1 mouse macrophages (9.2 × 106 cells containing 8.8 × 105 cpm in 0.5 ml of saline in each mouse) A: Plasma [3H]HDL and non-HDL cholesterol at 24 and 48 h. B: Liver [3H]cholesterol at 48 h. C: Fecal [3H]cholesterol and [3H]tracer from fecal bile acids over 48 h. Values are mean ± SEM. The amount of [3H]tracer was also expressed as a fraction of the injected dose.

Statistical methods

One-way ANOVA was used to compare differences among genotypes in macrophage-specific RCT experiments. Unpaired Student's *t*-test or Mann-Whitney U test were used to compare data obtained from T0901317-treated and untreated G5G8$^{+/+}$ and G5G8$^{-/-}$ mice. GraphPad Prism 4.0 software (GraphPad, San Diego, CA) was used to perform all statistical analyses. A *P* value <0.05 was considered statistically significant.

RESULTS

ABCG5/G8 deficiency does not impair reverse [³H]cholesterol transport from a macrophage cell line to feces in mice

G5/G8$^{-/-}$ mice fed the chow diet showed several previously reported phenotypes, such as increased mean plasma levels of β-sitosterol and campesterol and reduced plasma cholesterol levels (**Table 1**) (19, 23). Consistent with results of other groups (19, 23), a direct relationship was also observed between ABCG5 and ABCG8 gene dose and fecal neutral sterol excretion over a 3 day collection period (11.3 ± 0.9, 9.1 ± 1.4 and 7.0 ± 1.5 µmol/day·100 g body weight in G5G8$^{+/+}$, G5G8$^{+/-}$, and G5G8$^{-/-}$ mice, respectively; $P < 0.05$ between G5G8$^{-/-}$ and wild-type mice). Surprisingly, after intraperitoneal injection of [³H] cholesterol-labeled P388D1 mouse macrophages, plasma HDL-bound [³H]cholesterol levels of G5G8$^{-/-}$ mice were significantly higher than those of G5G8$^{+/+}$mice at 24 h (**Fig. 1A**). However, the [³H]tracer detected in liver, fecal cholesterol, and bile acids of G5G8$^{-/-}$ mice did not differ significantly from that of G5G8$^{+/+}$ mice (Fig. 1B, C). No sex-related differences were found in this experiment.

ABCG5 and -G8 are necessary for LXR-mediated induction of macrophage-specific RCT pathway

Similar to results obtained previously (19), LXR agonist T0901317 increased HDL cholesterol levels in G5G8$^{+/+}$ mice. As also reported previously (19), treatment with T0901317 decreased liver cholesterol content in G5G8$^{+/+}$ mice (**Table 2**). This was concomitant with the induction of fecal cholesterol excretion in T0901317-treated G5G8$^{+/+}$

mice, but not in G5G8$^{-/-}$ mice (Table 2). No differences were found in fecal bile acids between treated and non-treated animals in either of the two genotypes (Table 2). Our data were similar in both males and females and, therefore, were pooled for analysis.

We conducted a separate experiment to determine the fate of RCT from a macrophage cell line to feces in G5/G8$^{+/+}$ and G5/G8$^{-/-}$ mice treated with or without T0901317 (**Fig. 2**). T0901317 treatment did not affect plasma [³H]cholesterol levels at 24 h and 48 h in either mouse genotype (Fig. 2A). Liver [³H]cholesterol did not differ significantly between groups (Fig. 2B). However, the amount of [³H]fecal cholesterol was 2-fold higher in G5/G8$^{+/+}$ mice treated with T0901317 compared with un-treated G5/G8$^{+/+}$ mice, whereas [³H]fecal cholesterol levels were similar in untreated and T0901317-treated G5/G8$^{-/-}$ mice (Fig. 2C). The [³H]tracer detected in bile acids from T0901317-treated G5/G8$^{+/+}$ and G5/G8$^{-/-}$ mice did not differ significantly from that of untreated mice (Fig. 2C).

To determine the fate of [³H]cholesterol from the HDL core, untreated and T0901317-treated G5/G8$^{+/+}$ and G5/G8$^{-/-}$ mice were injected intravenously with HDL-[³H]cholesteryl oleate (**Fig. 3**). Plasma clearance of intra-venously injected [³H]HDL was significantly slower in T0901317-treated G5/G8$^{+/+}$ mice compared with un-treated mice (Fig. 3A). However, more than 90% of the [³H]cholesteryl oleate bound to HDL had been cleared from plasma at 48 h in all groups of mice (Fig. 3A). LXR activation did markedly increase the recovery of HDL-derived [³H]cholesterol in the feces of G5/G8$^{+/+}$ mice collected over 2 days (Fig. 3B). In contrast, LXR agonist failed to increase fecal [³H]cholesterol with an HDL origin in G5/G8$^{-/-}$ mice. No difference was found in [³H]tracer in fecal bile acids (Fig. 3B).

Effects of LXR agonist on small intestine and liver lipid-related gene expression in G5G8$^{+/+}$ and G5G8$^{-/-}$ mice

Small intestine and liver mRNA levels of known LXR target genes were determined in G5/G8$^{+/+}$ and G5/

TABLE 2. Liver lipids and fecal sterol excretion in ABCG5/G8$^{+/+}$ and ABCG5/G8$^{-/-}$ mice fed a chow diet treated with (+) or without (−) LXR agonist T0901317 for 7 days

	ABCG5/G8$^{+/+}$		ABCG5/G8$^{-/-}$	
	− T0901317	+ T0901317	− T0901317	+ T0901317
Liver weight (g)	1.21 ± 0.10	1.56 ± 0.14	1.41 ± 0.13	1.60 ± 0.10
Liver cholesterol (µmol/g liver)	4.8 ± 0.01	2.9 ± 0.2^a	0.3 ± 0.04	1.7 ± 0.1^a
Liver campesterol (µmol/g liver)	0.1 ± 0.02	Nd	0.2 ± 0.01	0.8 ± 0.04^a
Liver sitosterol (µmol/g liver)	0.1 ± 0.02	Nd	0.3 ± 0.05	1.9 ± 0.2^a
Fecal output (g/day)	0.75 ± 0.02	0.88 ± 0.10	0.84 ± 0.06	0.91 ± 0.14
Fecal neutral sterol output (µmol/day·100g bw)	9.1 ± 1.5	20.1 ± 0.7^a	7.4 ± 1.2	7.4 ± 0.2
Fecal cholesterol output (µmol/day·100g bw)	3.2 ± 1.1	15.8 ± 7.5^a	1.8 ± 0.5	3.5 ± 0.8
Fecal bile acid output (µmol/day·100g bw)	11.8 ± 2.1	11.5 ± 2.2	12.3 ± 1.7	12.7 ± 1.1

bw, body weight. Feces were collected over 3 consecutive days from five individual mice per group. An aliquot of 100 mg of liver and 1 g of feces from mice described in Table 1 was processed for determination of sterol content by GC-MS after lipid extraction. Bile acids of 1 g of feces were measured by the 3α-hydroxysteroid dehydrogenase method. Values are mean ± SEM of five individual animals per group.
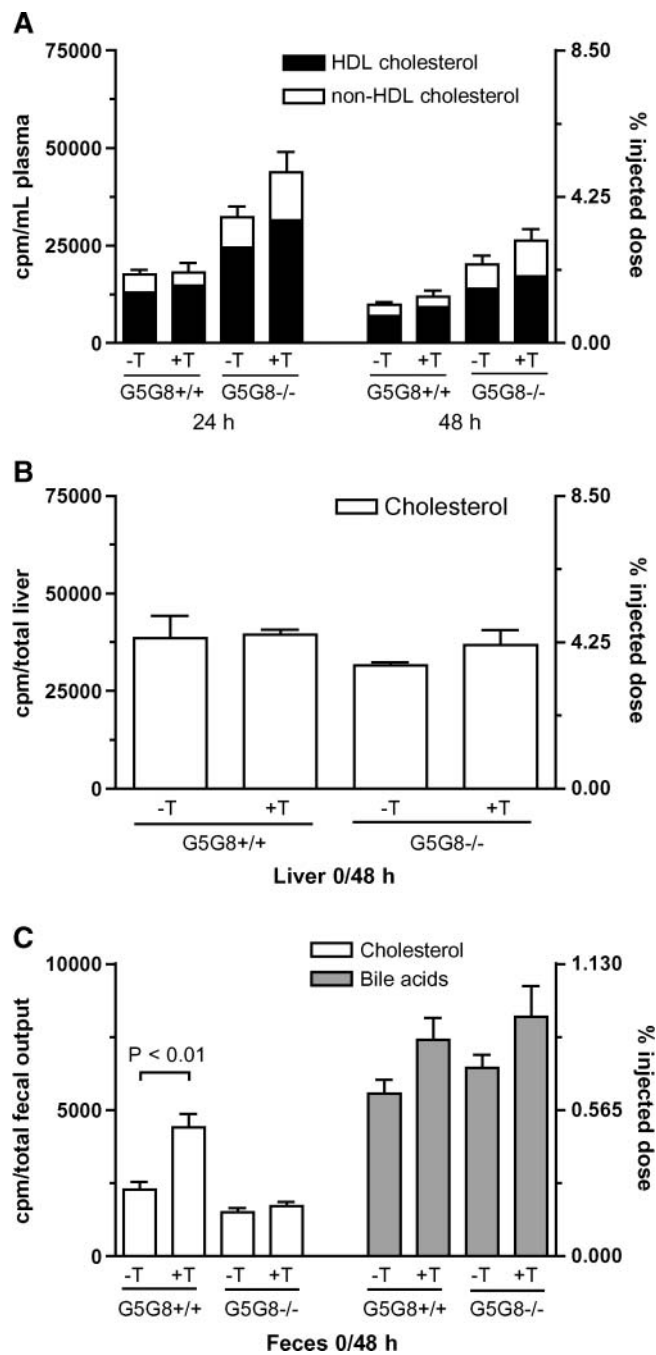^{a} $P < 0.05$ between T0901317-treated and vehicle-treated mice of each genotype.

Fig. 2. Reverse cholesterol transport from macrophages to feces in G5G8$^{+/+}$ and G5G8$^{-/-}$ mice treated with (+) or without (−) T0901317 for 7 days. Individually housed mice were injected intraperitoneally with [3H]cholesterol-labeled P388D1 mouse macrophages. A: Plasma [3H]HDL and non-HDL cholesterol at 24 and 48 h. B: Liver [3H]cholesterol at 48 h. C: Fecal [3H]cholesterol and [3H]tracer from fecal bile acids over 48 h. Values are mean ± SEM of seven mice (four males and three females in each group). The amount of [3H]tracer was also expressed as a fraction of the injected dose.

G8$^{-/-}$ mice (**Fig. 4**). Liver ABCG5 and ABCG8 mRNA expression was markedly increased upon LXR agonist treatment in G5/G8$^{+/+}$ mice (Fig. 4B). In contrast, intestinal ABCG5 and ABCG8 expression in treated G5/G8$^{+/+}$ mice tended to increase 1.5-fold, although it did not reach sta-
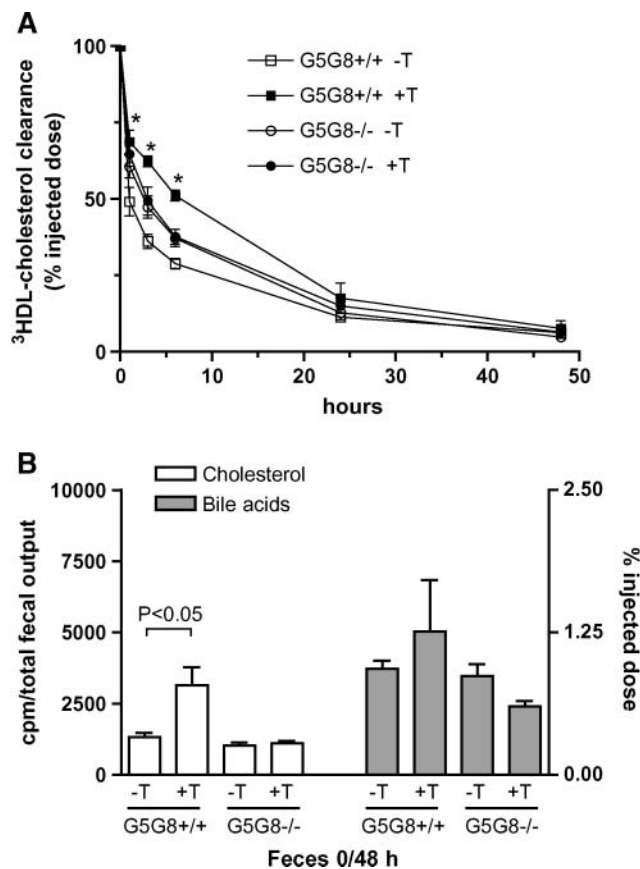

Fig. 3. In vivo clearance of [3H]cholesteryl oleate-radiolabeled HDL and excretion of tracer in fecal cholesterol and bile acids in G5G8$^{+/+}$ and G5G8$^{-/-}$ mice treated with (+) or without (−) T0901317. Individually housed mice were injected intravenously with 500,000 cpm of autologous [3H]HDL-cholesteryl oleate, and plasma radioactivity was measured over 48 h. A: Blood samples were taken at the indicated times, and [3H] activity was determined in plasma to draw plasma decay curves. B: Fecal [3H]cholesterol and [3H]tracer from fecal bile acids over 48 h. Values are mean ± SEM of six mice (three males and three females in each group). The amount of [3H]tracer was also expressed as a fraction of the injected dose. * Significant differences between T0901317-treated (+T) and vehicle-treated (−T) G5G8$^{+/+}$ mice are indicated ($P < 0.01$).

tistical significance compared with vehicle-treated G5/G8$^{+/+}$ mice (Fig. 4A). The T0901317-mediated induction of small intestine and liver ABCA1 and ABCG1 and liver sterol-regulatory element binding protein 1c (SREBP-1c) mRNA expression was similar in G5/G8$^{+/+}$ and G5/G8$^{-/-}$ mice (Fig. 4). Treatment with T0901317 was also associated with significant rises in mRNA levels of small intestine SREBP-1c and liver CYP7A1 in G5G8$^{+/+}$ mice. However, no significant changes in mRNA levels of these two genes upon LXR agonist treatment were found in G5/G8$^{-/-}$ mice (Fig. 4).

DISCUSSION

In this study, we show for the first time, to our knowledge, that ABCG5 and ABCG8 transporters are essential
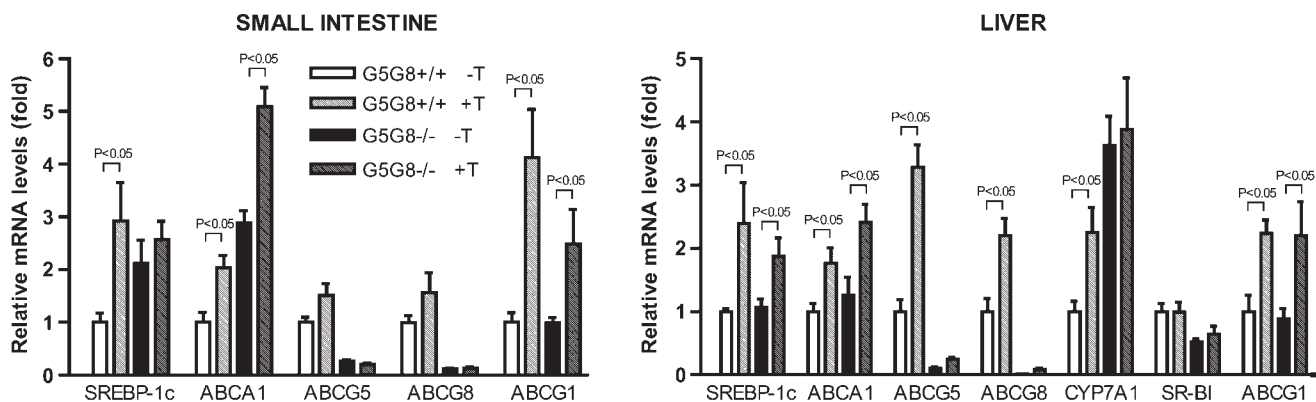
Fig. 4. Real-time RT-PCR quantification of relative mRNA expression in small intestines and livers of G5G8$^{+/+}$ and G5G8$^{-/-}$ mice treated with (+) or without (−) T0901317. Small intestines and livers were obtained from the mice described in Table 1. In mRNA expression, the signal of vehicle-treated ABCG5G8$^{+/+}$ mice was set to a normalized value of 1 arbitrary unit. Results are expressed as mean ± SEM of five individual animals per group (three males and two females in each group). For ABCG5G8$^{-/-}$ mice, putatively inactive mRNA was detectable in ABCG5/G8 mice, because the PCR primers are located outside the disrupted exons (63, 64). PGK1 was used as internal control. Values are mean ± SEM.

for the LXR agonist-mediated induction of RCT from a macrophage cell line to feces in vivo. Previous studies have shown that stimulation of cholesterol excretion by the administration of LXR agonist T0901317 required the expression of ABCG5 and ABCG8 (19), an observation confirmed in our study (Table 2). However, the contribution of specific cell sources to this increased fecal cholesterol output was not addressed (19). It should be noted that the fecal excretion of cholesterol that has its origin in macrophages is minimal compared with that of the remaining tissues (39). Therefore, the rate of hepatobiliary cholesterol or fecal cholesterol excretion does not necessarily reflect macrophage-specific RCT (14–17). This is indeed what we found in the present study in which ABCG5/G8$^{-/-}$ mice presented a 20% to 40% decrease in fecal neutral sterol excretion but unchanged RCT from a macrophage cell line to feces in vivo. These findings are, however, somewhat surprising, considering that disruption of ABCG5/G8 resulted in a marked reduction in biliary cholesterol levels (23, 40). This could indicate an important role for the intestine in the model used in this study. It is interesting to note that recent data suggest that the intestine may play an important role in excreting cholesterol from plasma to the intestinal lumen and feces (41). Thus, it is possible that the intestine could promote a direct transport of HDL-bound [^{3}H]cholesterol into the enterocyte by ABCG5- and -G8-independent routes, thus contributing to the relatively low change in fecal neutral sterol excretion and the unaltered RCT from macrophage to feces in G5G8$^{-/-}$ mice.

The magnitude of the increase in macrophage-derived fecal [^{3}H]cholesterol excretion in GW3965- and T0901317-treated mice was not associated with a similar increase in plasma and liver [^{3}H]cholesterol [Fig. 2 and (29)]. Importantly, changes in plasma, HDL, and liver cholesterol tracer did not correlate with total cholesterol mass, and the apparent specific activity of liver markedly differed from that of plasma (Fig. 2 and Table 1). Thus, our data indicate that

once effluxed from the macrophages, the flux of [^{3}H]cholesterol through plasma and liver compartments did not seem to be influenced by total plasma, HDL, and liver cholesterol mass. Of note, liver cholesterol homeostasis is altered in ABCG5/G8-deficient mice (Table 2), presumably as a consequence of interference in SREBP-2 cleavage by stigmasterol (42). Therefore, although our main conclusions were based only on measurements of tracer, we believe that tracer was tracking the flux of macrophage-derived cholesterol mass.

Our results indicate that the major effect of the LXR agonist accelerating the rate of fecal [^{3}H]cholesterol excretion involves liver ABCG5/G8 upregulation. Importantly, treatment with T0901317 may reduce fractional cholesterol absorption in G5G8$^{+/+}$ mice (19) and, thus, contribute to the increased fecal [^{3}H]cholesterol excretion and, consequently, RCT from macrophages to feces.

An intriguing observation was the decreased plasma clearance of labeled HDL injected into the LXR-treated G5G8$^{+/+}$ mice, although this coexisted with increased [^{3}H]cholesterol in feces (Fig. 3). The causes of this effect are unknown, but it is possible that the enlargement of HDL via LXR activation (43) could reduce, at least in part, the liver cholesterol selective uptake (44).

Gene expression pattern induction by LXR treatment was, in general, similar to the results reported by other groups (19, 29). Importantly, accumulation of some plant sterols in these mice, particularly those with an unsaturation within the side chain, can activate LXRα (45), and this could explain the normal or even increased expression of several LXR target genes in our G5G8$^{-/-}$ mice. Thus, we found a significant increase in liver CYP7A1 in G5G8$^{-/-}$ mice, and this could avoid, at least in part, the LXR-mediated increase in the expression of this gene in G5G8$^{-/-}$ mice. In any event, T0901317 treatment did not affect fecal [^{3}H]bile acid excretion in either genotype, despite upregulation of liver CYP7A1 in LXR agonist-treated G5G8$^{+/+}$ mice. Our data concur with the finding

that GW3965-treated C57BL/6 and human apoB/CETP double-transgenic mice showed unchanged fecal [³H]bile acid excretion despite the increased fecal macrophage-derived [³H]cholesterol (29). One possible mechanism contributing to this effect is the high turnover of bile acids in mice (30).

In conclusion, our results demonstrate that the presence of ABCG5/G8 transporters is required for LXR-mediated induction of RCT from macrophages to feces in vivo, and support the hypothesis that these transporters play a key role in the final step of this pathway. These data also suggest that upregulation of ABCG5/G8 may be an effective strategy to increase macrophage-specific RCT pathway and reduce atherosclerosis (46, 47).

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