Overexpression and deletion of phospholipid transfer protein reduce HDL mass and cholesterol efflux capacity but not macrophage reverse cholesterol transport[S]

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Abstract **Phospholipid transfer protein (PLTP) may affect macrophage reverse cholesterol transport (mRCT) through its role in the metabolism of HDL. Ex vivo cholesterol efflux capacity and in vivo mRCT were assessed in PLTP deletion and PLTP overexpression mice. PLTP deletion mice had reduced HDL mass and cholesterol efflux capacity, but unchanged in vivo mRCT. To directly compare the effects of PLTP overexpression and deletion on mRCT, human *PLTP* was overexpressed in the liver of wild-type animals using an adeno-associated viral (AAV) vector, and control and PLTP deletion animals were injected with AAV-null. PLTP overexpression and deletion reduced plasma HDL mass and cholesterol efflux capacity. Both substantially decreased ABCA1-independent cholesterol efflux, whereas ABCA1-dependent cholesterol efflux remained the same or increased, even though preβ HDL levels were lower. Neither PLTP overexpression nor deletion affected excretion of macrophage-derived radiocholesterol in the in vivo mRCT assay. The ex vivo and in vivo assays were modified to gauge the rate of cholesterol efflux from macrophages to plasma. PLTP activity did not affect this metric. Thus, deviations in PLTP activity from the wild-type level reduce HDL mass and ex vivo cholesterol efflux capacity, but not the rate of macrophage cholesterol efflux to plasma or in vivo mRCT.—** Kuwano, T., X. Bi, E. Cipollari, T. Yasuda, W. R. Lagor, H. J. Szapary, J. Tohyama, J. S. Millar, J. T. Billheimer, N. N. Lyssenko, and D. J. Rader. **Overexpression and deletion of phospholipid transfer protein reduce HDL mass and cholesterol efflux capacity but not macrophage reverse cholesterol transport. *J. Lipid Res.* 2017.** 58: **731–741.**

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Phospholipid transfer protein (PLTP) facilitates bidirectional exchange of lipid between lipoproteins and performs many extra- and intra-cellular functions (1). In the plasma, it mediates a net transfer of phospholipid from triglyceride-rich apoB-lipoprotein to HDL and induces fusion of smaller-sized HDL particles into larger-sized HDL particles with concurrent release of lipid-poor apoA-I (1). In humans, common variants at the PLTP locus are strongly associated with plasma HDL cholesterol (HDL-C) levels (2). Interestingly, both overexpression and deletion of PLTP in the mouse lead to a decrease in HDL-C (3, 4).

Reverse cholesterol transport from macrophages (mRCT) to the liver for excretion in feces is thought to reduce atherosclerosis independently of HDL-C (5, 6). The ex vivo HDL-C efflux capacity and in vivo mRCT assays have been used to evaluate how various factors of interest affect mRCT (5, 7). Little data are available regarding the effect of PLTP on these measures of HDL function. Whole-body overexpression of PLTP in the mouse has been reported to reduce macrophage cholesterol excretion by possibly increasing cholesterol uptake in the intestine in the in vivo mRCT assay (8). To gain a better understanding of the role of PLTP in mRCT, we assessed cholesterol efflux capacity and in vivo mRCT in the contexts of PLTP liver-specific overexpression and whole-body deletion. The results show that plasma PLTP

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Abbreviations: AAV, adeno-associated virus; acLDL, acetylated LDL; ASCVD, atherosclerotic CVD; HDL-C, HDL cholesterol; hPLTP, human phospholipid transfer protein; hPLTP-tg, human phospholipid transfer protein transgenic; mRCT, macrophage reverse cholesterol transport; PLTP-KO, phospholipid transfer protein knockout; PLTP-Het, heterozygous for the *Pltp* targeted deletion allele; PLTP, phospholipid transfer protein.

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affects HDL mass and cholesterol efflux capacity, but not in vivo mRCT.

MATERIALS AND METHODS

Mice

C57BL/6J and B6.129P2-Pltp^{tm1Jia}/J (4) mice were acquired from the Jackson Laboratory and bred to derive wild-type controls, animals heterozygous for the *Pltp* deletion allele (PLTP-Het), and animals homozygous for the *Pltp* deletion allele (PLTP-KO). Animal genders, ages, and numbers in each experiment are indicated in the Results. All animal procedures were conducted in conformity with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and were approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

AAV vectors

The adeno-associated viral (AAV) vector, AAV.2/8.TBG.hPLTP, was used to overexpress human *PLTP* (AAV-hPLTP) in PLTP overexpression (hPLTP) mice. AAV.2/8TBG.PI.null.bGH (AAV-null) was employed as a negative control. The vectors were produced at the Penn Vector Core.

PLTP activity, plasma lipids, and size exclusion chromatography

PLTP activity was measured using Roar PLTP activity assay kits (Roar Biomedical). HDL-C and plasma phospholipid were measured on a Cobas Mira (Roche) biochemistry analyzer using EZ HDL Cholesterol (Trinity Biotech) and Phospholipids C (Wako) kits, respectively. For size exclusion chromatography, mouse plasma was resolved on two Superose 6 16/300 GL columns (GE Healthcare) connected in tandem.

Plasma cholesterol efflux capacity from macrophages

J774 macrophage cells were obtained from American Type Culture Collection and maintained in RPMI/10% FBS at 37°C in 5% CO_2. To measure cholesterol efflux capacity, J774 cells were seeded, allowed to attach for 6–24 h, incubated with 2 μCi/ml [1,2-^{3}H(N)]cholesterol or 0.12 μCi/ml [4-^{14}C]cholesterol (both from PerkinElmer) in RPMI/0.2% BSA (fatty acid free) or RPMI/2.5% FBS for 24 h. In most cases, the labeling medium also contained 25 μg/ml of acetylated LDL (acLDL) to load the cells with cholesterol. The cells were then treated with 0.3 mM 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate sodium salt (cAMP; Sigma-Aldrich) in RPMI/0.2% BSA for 16 h to upregulate ABCA1 expression and exposed to mouse plasma (0.5–5% in RPMI/0.1% BSA/ +/− 0.15 mM cAMP or RPMI/ +/− 0.3 mM cAMP medium) or medium without plasma for 4 h. Mouse plasma was not apoB-lipoprotein depleted because it contains little apoB-lipoprotein in comparison with human plasma. Cell medium was centrifuged to remove floating cells; cell lipids were extracted with isopropanol or hexane/isopropanol (3:2, v/v), and both medium and cell lipids were read in a scintillation counter. Cell cholesterol efflux was quantified as a percentage of counts in the medium relative to the total counts in the medium and cells. The average percent efflux to cell medium without plasma was subtracted from percent efflux values to medium with plasma. Probucol (20 μM) or vehicle (0.2% DMSO) was applied after [^{3}H]cholesterol labeling and before plasma addition for 2 h, then cholesterol efflux to plasma was allowed to proceed in the presence of probucol.

In vivo mRCT assays

In vivo mRCT assays were conducted as previously described (9). J774 cells were incubated with 3–5 μCi/ml [^{3}H]cholesterol and 25–100 μg/ml acLDL in RPMI/0.2% BSA or RPMI/5% FBS for 48 h, washed with PBS, and further kept in RPMI/0.2% BSA for 4–6 h. The cells were then scraped, centrifuged, resuspended in RPMI, and injected into the mouse peritoneum. Each mouse received 3.7×10^6 cells and 8.4×10^6 cpm in 400 μl of medium (PLTP deletion experiment), 4×10^6 cells and 2.0×10^6 cpm in 300 μl of medium (first direct comparison of PLTP overexpression and deletion experiment), or 5.0×10^6 cells and 1.7×10^7 cpm in 500 μl of medium (second direct comparison experiment). Blood was collected from the retro-orbital plexus at 6, 24, and 48 h after the cell injection in the PLTP deletion and second direct comparison experiments. In the first direct comparison experiment, 25 μl of blood was collected from the tail vein at 0.5, 1, 1.5, 2, 4, and 24 h after the cell injection; the terminal 48 h bleed was conducted from the retro-orbital plexus. Blood was centrifuged at 10,000 *g* at 4°C for 10 min; plasma was collected and read in a scintillation counter. The formed elements fraction was solubilized with SOLVABLE (PerkinElmer) as recommended by the manufacturer and then read in a scintillation counter. Mice were housed individually in wire-bottom cages during the 48 h period after the cell injection to collect feces. Feces were weighed, soaked in water (at 100 mg/ml) overnight at 4°C, combined with an equal volume of ethanol, and homogenized. Aliquots of the homogenate were either diluted two times with ethanol or solubilized with SOLVABLE (PerkinElmer) and then read in a scintillation counter. Mice were euthanized; the liver was perfused with cold PBS and collected. Liver lipids were extracted by the Bligh and Dyer method and read in a scintillation counter; or liver tissue was homogenized in PBS with a steel bead using TissueLyser II (Qiagen) and read in a scintillation counter. Bile was collected and read in a scintillation counter without processing. Radiocholesterol counts in plasma, formed elements, liver, bile, and feces were expressed as a percent of the counts injected with the cells.

Agarose and polyacrylamide gel electrophoresis

Plasma was analyzed on 0.7% agarose gels in 0.05 M barbital buffer (pH 8.6)/0.33% albumin (w/v) as previously described (10) and on native 4–20% polyacrylamide gels in Tris-glycine buffer (WedgeWell precast gels from Thermo Fisher). Agarose and polyacrylamide gels were blotted and probed with an anti-apoA-I antibody (K23001R; Meridian Life Science, formerly BioDesign).

Statistics

GraphPad Prism (GraphPad Software) was used to graph and analyze data as indicated in the figure legends.

RESULTS

PLTP deletion reduces cholesterol efflux capacity, but not in vivo mRCT

To determine the effect of PLTP deletion on HDL metabolism and the metrics of mRCT, fasting plasma was collected from wild-type, PLTP-Het, and PLTP-KO mice (n = 8, 8, and 6, respectively; all males in the wild-type and PLTP-Het groups, and four males and two females in the PLTP-KO group; all 12 weeks old) and analyzed for PLTP activity, HDL-C, and plasma phospholipid. PLTP-Het and PLTP-KO mice exhibited, respectively, 23 ± 9% and 87 ± 1% reduced PLTP activity (**Fig. 1A**), 11 ± 5% and 70 ± 5%

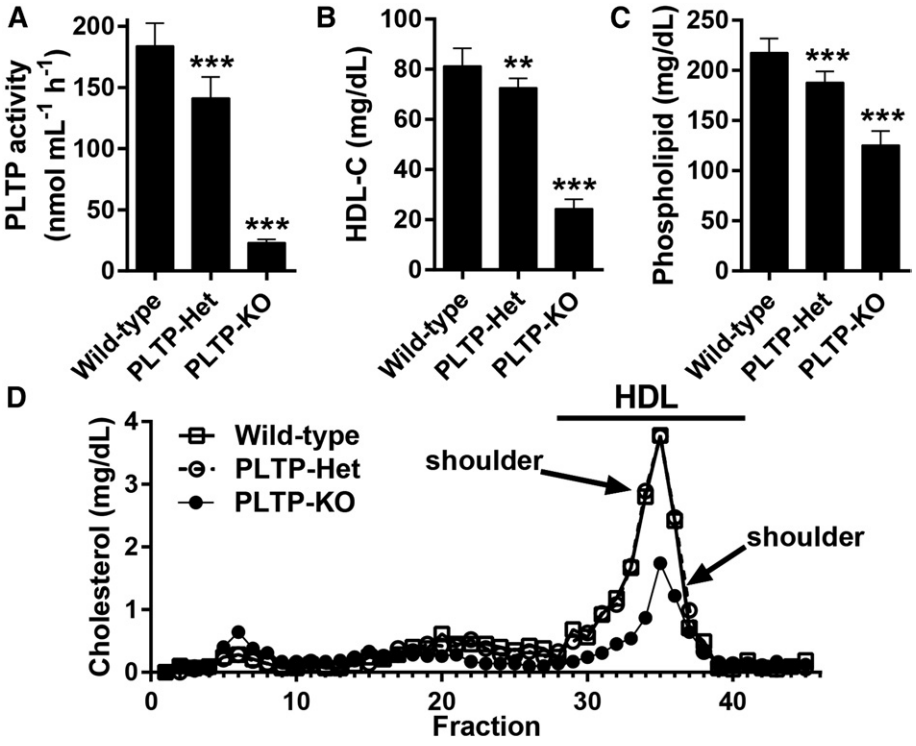

Fig. 1. Effects of PLTP deletion on HDL mass. A–C: PLTP activity, HDL-C, and phospholipid in plasma of wild-type mice, animals heterozygous for PLTP deletion (PLTP-Het), and animals homozygous for PLTP deletion (PLTP-KO). Data are expressed as mean ± SD. Statistical analysis, one-way ANOVA with wild-type values taken as the control for Bonferroni's multiple comparisons test (**$P < 0.01$; ***$P < 0.001$). D: Plasma lipid profile of wild-type and PLTP-deficient mice. Arrows indicate shoulder regions at the HDL peak.

lower HDL-C (Fig. 1B), and 13.6 ± 5% and 42.3 ± 7% lower plasma phospholipid (Fig. 1C). Mouse plasma was pooled by genotype and analyzed by size exclusion chromatography. The centers of the wild-type, PLTP-Het, and PLTP-KO HDL peaks eluted in the same fraction (Fig. 1D). The wild-type and PLTP-Het HDL peaks had a shoulder extending to the larger-sized elution fractions, while the PLTP-KO HDL peak had a shoulder extending to the smaller-sized elution fractions. This suggests that PLTP-KO HDL may include particle species that are smaller in size than the wild-type HDL.

To assess the effect of PLTP deletion on cholesterol efflux capacity, pooled mouse plasma was exposed to acLDL-loaded J774 macrophage cells that were treated with cAMP to induce expression of ABCA1 or with vehicle. Total cholesterol efflux from cAMP-treated cells (i.e., cholesterol efflux capacity) to PLTP-Het and PLTP-KO plasma was lower than to wild-type plasma by 11 ± 2% ($P = 0.004$) and 17 ± 4% ($P = 0.0004$), respectively (**Fig. 2A**). Cholesterol efflux from untreated J774 cells (i.e., ABCA1-independent efflux; Fig. 2B) to plasma of PLTP-Het animals was reduced by 13 ± 3% (not significant) and to plasma of PLTP-KO mice by 51 ± 3% ($P < 0.0001$). ABCA1-dependent efflux (i.e., the difference in efflux between cAMP-treated and untreated cells) was significantly increased by 225 ± 31% to plasma from PLTP-KO mice (Fig. 2C). These data indicate that PLTP deletion results in reduced ABCA1-independent efflux, but increased ABCA1-dependent efflux.

The same mice were subsequently injected with acLDL-loaded [^{3}H]cholesterol-labeled J774 cells into the peritoneum for assessment of in vivo mRCT. Plasma was collected at 6, 24, and 48 h after the cell injection. Plasma levels of [^{3}H]cholesterol were markedly reduced in PLTP-KO mice at all sampling points (**Fig. 3A**). Feces were collected continuously during the 48 h period; at 48 h, the mice were euthanized to collect liver and bile. The liver [^{3}H]cholesterol counts were moderately, but significantly, lower in PLTP-KO, but not PLTP-Het mice, in comparison with wild-type animals (Fig. 3B). However, bile (Fig. 3C) and fecal sterol counts (Fig. 3D) were not significantly different among the PLTP genotypes. Thus, PLTP deletion does not significantly affect mRCT despite a major reduction in cholesterol efflux capacity.

Direct comparison of PLTP overexpression and deletion with respect to cholesterol efflux capacity and mRCT

PLTP overexpression has also been reported to reduce HDL-C and to lack an effect on the transport of macrophage cholesterol to liver (8). To elucidate any differences between the PLTP overexpression and PLTP deletion phenotypes with respect to macrophage cholesterol transport, an experiment was carried out to directly compare the effects of PLTP liver-specific overexpression and whole-body deletion on cholesterol efflux capacity and in vivo mRCT. Wild-type mice (n = 7) were injected with 3×10^{10} genome copies of AAV-hPLTP to overexpress human PLTP in the

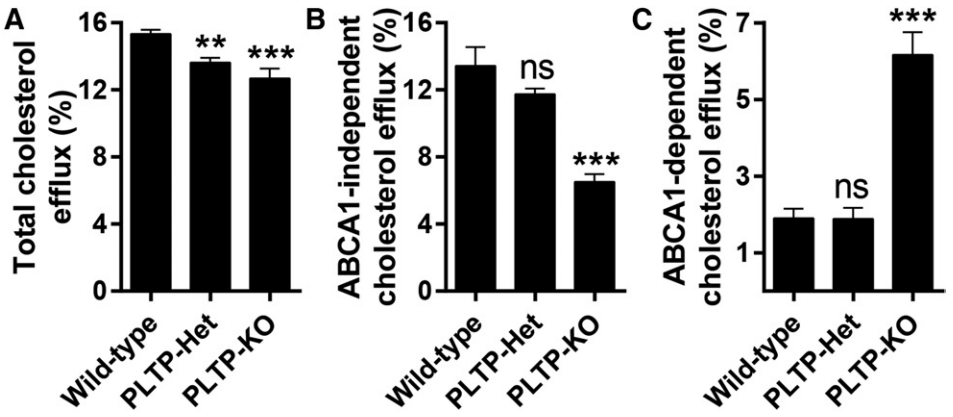

Fig. 2. Cholesterol efflux capacity of plasma from PLTP-deficient mice. Plasma from wild-type, PLTP-Het, and PLTP-KO animals was pooled by genotype and exposed to cAMP-treated, i.e., ABCA1-upregulated, or vehicle-treated J774 macrophage cells. A: Efflux from cAMP-treated cells represents cholesterol efflux capacity. B: Efflux from vehicle-treated cells represents ABCA1-independent efflux by diffusion-based pathways. C: The difference between efflux from cAMP-treated and vehicle-treated cells represents ABCA1-dependent efflux. Data are expressed as mean ± SD (n = 4). Statistical analysis, one-way ANOVA with wild-type values set as the control for Bonferroni's multiple comparisons test (**$P < 0.01$; ***$P < 0.001$; ns, not significant).

liver. The reference group of animals (n = 7) consisted of wild-type mice injected with 3×10^{10} genome copies of a control AAV-null vector; PLTP-KO mice (n = 6) were also injected with 3×10^{10} genome copies of the AAV-null control vector to allow for direct comparison to the PLTP overexpression animals (all males, 8–16 weeks old, age-matched among the groups). HDL-C and plasma phospholipid were measured before and 14 and 28 days after the virus injection in fasting plasma (4 h). At the 28 day mark, PLTP overexpression and deletion plasmas contained, respectively, 81 ± 9% and 73 ± 9% less HDL-C and 68 ± 8% and 51 ± 5% less phospholipid than wild-type plasma (**Fig. 4A**). The 28 day plasma was pooled by genotype/AAV type and used to measure PLTP activity. PLTP activity in the plasma of PTLP overexpression mice was higher than in wild-type plasma by 27 ± 6% ($P = 0.0002$); PLTP activity in PLTP deletion plasma was negligible (Fig. 4B).

Cholesterol efflux capacity was measured in the 28 day pooled plasma. Total cholesterol efflux to PLTP overexpression and deletion plasmas was reduced by 41 ± 7% and 41 ± 13%, respectively; ABCA1-independent efflux was reduced by 67 ± 2% and 63 ± 6%, respectively; ABCA1-dependent efflux was elevated by 30 ± 12% (not significant) and remained unchanged, respectively (**Fig. 5A–C**). To determine whether the decrease in cholesterol efflux capacity resulted mainly from a loss of HDL mass, the amount of plasma added to cells in a second set of cholesterol efflux assays was adjusted to the same amount of HDL-C (1.6 μg of HDL-C per well of a 24-well plate or 0.5, 2.6, and 1.9% of wild-type, PLTP overexpression, or PLTP deletion plasma, respectively, in cell medium). HDL-C-normalized ABCA1-independent efflux to PLTP overexpression and deletion plasma was elevated modestly by 33 ± 4% and 21 ± 7% (both not significant), respectively, while HDL-C-normalized ABCA1-dependent efflux rose more dramatically by 93 ± 15% and 83 ± 7%, respectively, and HDL-C-normalized cholesterol efflux capacity (i.e., total efflux) was elevated by 60 ± 7% and 49 ± 2%, respectively (Fig. 5D–F). These observations suggest that PLTP overexpression and deletion reduce cholesterol efflux capacity by lowering the ability of plasma to accept cholesterol by ABCA1-independent diffusional efflux, while the ability of plasma to accept cholesterol by the ABCA1-dependent pathway remains unaltered or trends toward an increase.

Preβ HDL is a key acceptor of cell cholesterol released by the ABCA1-depedent pathway (11). Plasma was resolved on an agarose gel, blotted, and probed with an anti-apoA-I antibody to ascertain whether PLTP activity affected preβ HDL levels. PLTP overexpression and deletion dramatically reduced the amounts of preβ HDL and α HDL (**Fig. 6A**). Because HDL was detected using an anti-apoA-I antibody, this outcome implies a dramatic loss of apoA-I. Plasma analysis using native polyacrylamide gel electrophoresis confirmed the loss of HDL/apoA-I in the mice with altered PLTP expression (Fig. 6B). HDL from PLTP deletion mice also exhibited significant changes in the HDL particle species assortment: in addition to the main species prominently present in HDL from all three groups of mice, PLTP deletion HDL also contained two smaller species (one very prominent and the other near the limit of detection) that were absent in the wild-type and PLTP overexpression HDL. These results show that preβ HDL was reduced together with α HDL and thus cannot account for the preservation of ABCA1-dependent efflux in PLTP overexpression and deletion plasma.

The same mice were injected with acLDL-loaded [^{3}H] cholesterol-labeled J774 cells into the peritoneum on day 32 after the vector administration. The [^{3}H]cholesterol counts were significantly lower in plasma from PLTP overexpression mice by the 1.5 h sampling point and in plasma from PLTP deletion mice by the 4 h sampling point (**Fig. 7A**). At the 24 and 48 h marks after the cell injection, [^{3}H]cholesterol counts were reduced by 60 ± 9% and 70 ± 10%, respectively, in PLTP overexpression plasma and by 37 ± 20% and 58 ± 11%, respectively, in PLTP deletion plasma (Fig. 7A). Notwithstanding the reduced plasma

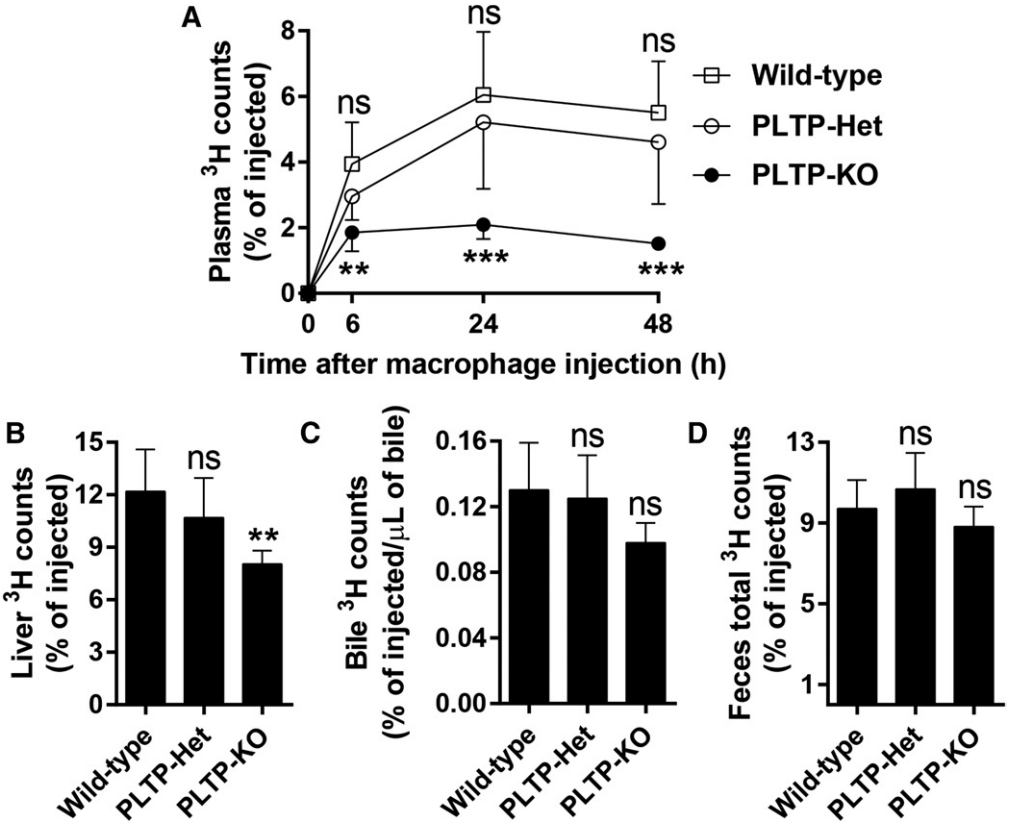

Fig. 3. mRCT in PLTP-deficient mice. acLDL-loaded and [^{3}H]cholesterol-labeled (not treated with cAMP) J774 macrophage cells were injected into the peritoneum. Plasma was sampled from the retro-orbital plexus at the indicated time points. A: Plasma ^{3}H counts were quantified as a percent of the total injected [^{3}H]cholesterol. B, C: After 48 h, mice were euthanized to measure liver and bile ^{3}H counts. D: During the 48 h period, mice were kept in individual wire-bottom cages to collect feces for measuring fecal sterols. Data are expressed as mean ± SD. Statistical analysis in (A), one-way ANOVA on each time point with Bonferroni's multiple comparisons test to compare wild-type values with PLTP-Het and PLTP-KO values (**$P < 0.01$; ***$P < 0.001$; ns, not significant). Statistical analysis in (B–D), one-way ANOVA with wild-type values set as the control for Bonferroni's multiple comparisons test [P values same as in (A)].

radiolabel counts, the liver, bile, and feces [^{3}H]cholesterol counts remained unchanged (Fig. 7B–D), indicating that the total amount of cholesterol transported to the liver was unaffected. The [^{3}H]cholesterol was read in the 48 h blood cell fractions (i.e., the pellet of red and white blood cells and platelets that forms during blood centrifugation and together with plasma comprises whole blood; this fraction is called the formed elements of blood). Formed elements of wild-type, PLTP overexpression, and PLTP deletion animals contained similar amounts of the radiolabel (Fig. 7E). Interestingly, the wild-type, PLTP overexpression, and PLTP deletion formed elements fractions contained the same percentage of the injected [^{3}H]cholesterol as the wild-type plasma (1.4 ± 0.5%, 1.3 ± 0.5%, and 1.6 ± 0.5%, respectively, in the formed elements versus 1.4 ± 0.3% in the wild-type plasma). These findings show that PLTP activity does not affect mRCT or cholesterol levels in the formed elements fraction.

To determine whether mouse manipulation during the mRCT assay affected PLTP activity or plasma properties, the 48 h plasma was pooled by genotype/AAV type and assessed for PLTP activity, HDL particle composition, and cholesterol efflux capacity. PLTP activity in the wild-type 48 h plasma was essentially the same as in the wild-type 28 day plasma (229 ± 5 nmol ml^{-1} h^{-1} for 48 h plasma versus 213 ± 1 nmol ml^{-1} h^{-1} for 28 day plasma). However, PLTP activity in the PLTP overexpression 48 h plasma was higher than in the PLTP overexpression 28 day plasma (333 ± 16 nmol ml^{-1} h^{-1} versus 271 ± 13 nmol ml^{-1} h^{-1}, respectively). The 48 h values for PLTP activity in the PLTP overexpression plasma were 45 ± 7% higher than in wild-type plasma (versus 27 ± 6% higher for the 28 day plasmas; see above). The 48 h values for PLTP activity in the PLTP deletion plasma were negligible. The 48 h plasma was analyzed using native polyacrylamide gel electrophoresis. PLTP overexpression 48 h plasma appeared to contain even less HDL/apoA-I than PLTP overexpression 28 day plasma (supplemental Fig. S1). However, in terms of HDL particle assortment, 48 h and 28 day plasmas of the same genotype/AAV type were similar (note that 48 h plasma was not fasting, while 28 day plasma was fasting). Total and ABCA1-independent cholesterol efflux from acLDL-loaded J774 cells to pooled PLTP overexpression 48 h plasma were reduced by 25 ± 4% and 55 ± 1%, respectively; ABCA1-dependent efflux to the same plasma was unaffected (supplemental Fig. S2). HDL-C-normalized ABCA1-independent

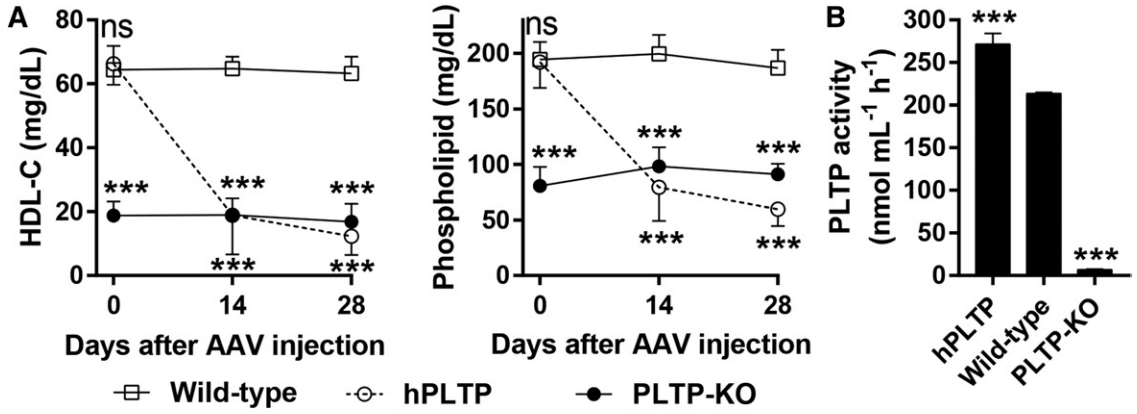

Fig. 4. Direct comparison of PLTP overexpression and deletion: HDL-C, plasma phospholipid, and PLTP activity. A: HDL-C and plasma phospholipid levels in wild-type, PLTP overexpression (hPLTP), and PLTP-KO animals at the indicated time points after the AAV-hPLTP injection. Data are expressed as mean ± SD. Statistical analysis, one-way ANOVA by time point with Bonferroni's multiple comparisons test to compare wild-type values with hPLTP and PLTP-KO values (***$P < 0.001$; ns, not significant). B: PLTP activity in pooled plasma from wild-type, PLTP overexpression, and PLTP deletion mice 28 days after the AAV-hPLTP vector injection. Representative results (n = 3). Data are expressed as mean ± SD. Statistical analysis, one-way ANOVA with Bonferroni's multiple comparisons test to compare wild-type values with hPLTP and PLTP-KO values (***$P < 0.001$; ns, not significant).

cholesterol efflux to PLTP overexpression and deletion 48 h plasma was elevated by 37 ± 2% and 26 ± 6%, respectively, while HDL-C-normalized ABCA1-dependent efflux was increased by 52 ± 10% and 44 ± 16%, respectively, and total cholesterol efflux rose by 47 ± 5% and 40 ± 8%, respectively. Thus, PLTP activity was elevated and HDL/apoA-I was reduced at the conclusion of mRCT assay relative to several days prior to the assay onset in PLTP overexpression mice, but these changes failed to dramatically change plasma cholesterol efflux capacity.

The direct comparison of the PLTP overexpression and deletion experiment was repeated with a second cohort of mice (n = 6 for all groups, all males, 5–15 weeks old, age-matched among the groups, injected with either 3×10^{10} genome copies of AAV-hPLTP or 3×10^{10} genome copies of AAV-null, as in the first comparison experiment). ABCA1-dependent cholesterol was measured in this experiment using probucol, an inhibitor of ABCA1 activity (12). The results were similar (supplemental Table S1), except the reductions in cholesterol efflux capacity and ABCA1-independent efflux were less severe in comparison with the first experiment (>40% and >60% in the first direct comparison versus <20% and <50% in the second direct comparison for cholesterol efflux capacity and ABCA1-independent efflux, respectively), and the change in ABCA1-dependent efflux was more dramatic. Overall the outcome in this second mouse cohort confirmed the findings in the first cohort: PLTP overexpression and deletion reduced cholesterol efflux capacity and plasma levels of macrophage-derived cholesterol, but not in vivo mRCT.

DISCUSSION

We employed a unique experimental design to directly compare the effects of increased and decreased PLTP activity on plasma HDL metabolism and metrics of mRCT in mice. The relationships between PLTP activity and HDL mass and between PLTP activity and plasma cholesterol efflux capacity, an ex vivo metric of mRCT, are parabolic (∩-shaped). The wild-type PLTP activity supports the highest HDL mass (i.e., the highest HDL-C, plasma phospholipid, apoA-I, preβ HDL, and α HDL levels) and plasma cholesterol efflux capacity, while PLTP overexpression- and deletion-induced deviations up or down from the wild-type activity level reduce HDL mass and cholesterol efflux capacity. Notwithstanding the effect on HDL metabolism and cholesterol efflux capacity, PLTP activity paradoxically plays no role in in vivo mRCT.

PLTP overexpression and deletion both reduce cholesterol efflux capacity by decreasing ABCA1-independent cholesterol efflux and leave ABCA1-dependent cholesterol efflux intact. Lower ABCA1-independent efflux stems from HDL loss. Lower levels of preβ HDL, a major cholesterol acceptor by the ABCA1-dependent pathway (11, 13), in PLTP overexpression and deletion plasma should have reduced ABCA1-dependent efflux. But this did not occur. One possibility is that ABCA1-dependent efflux to α (mature) HDL substitutes for ABCA1-dependent efflux to preβ HDL. PLTP has been shown to transfer cholesterol from J774 cells with upregulated ABCA1 expression to mature (ultracentrifugation isolated) HDL (14). Increased PLTP-mediated transfer of cholesterol to mature HDL could substitute for efflux to preβ HDL in PLTP overexpression plasma. ABCA1 has been shown to mediate cholesterol release directly to the smaller-sized HDL3b and HDL3c species (15). PLTP deletion plasma contains two abnormal smaller-sized HDL species. Efflux to these new species could account for the preservation of ABCA1-dependent efflux in PLTP deletion plasma.

In search of an explanation for the difference in the effects of PLTP activity on ex vivo plasma cholesterol efflux capacity and in vivo mRCT, we considered in detail the design of the two assays. Both assays employ J774 macrophage cells as a standardized source of cholesterol. To assess the net cholesterol flux from macrophages to plasma (instead

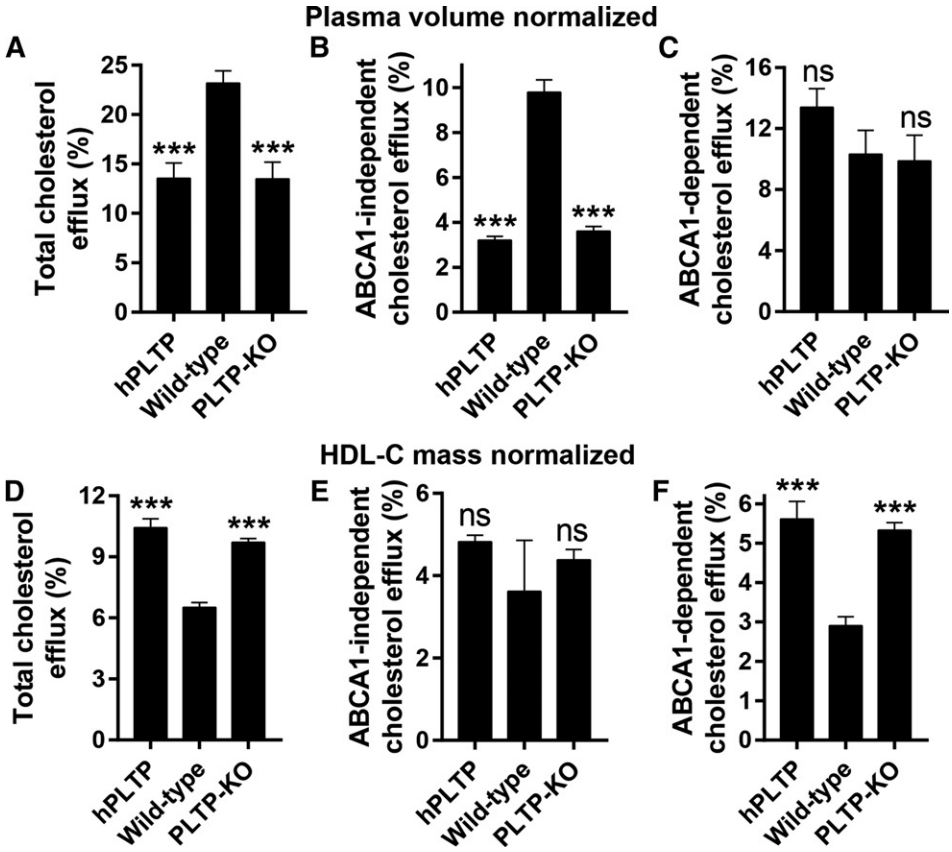

Fig. 5. Direct comparison of PLTP overexpression and deletion plasma with respect to cholesterol efflux capacity. Mouse pooled plasma was exposed to J774 macrophage cells treated with either cAMP to upregulated ABCA1 or vehicle. A–C: The same volume of plasma (1% of the cell medium) was added to cells regardless of plasma HDL-C concentration. D–F: The amount of plasma added to cells was adjusted to the same HDL-C amount (1.6 μg of HDL-C per well of a 24-well plate or 0.5, 2.6, and 1.9% of wild-type, PLTP overexpression, and deletion plasma, respectively, in cell medium). A, D: Total cholesterol efflux (i.e., cholesterol efflux capacity) and HDL-C-normalized total cholesterol efflux. B, E: ABCA1-independent (i.e., diffusional) efflux and HDL-C-normalized ABCA1-independent efflux. C, F: ABCA1-dependent and HDL-C-normalized ABCA1-dependent efflux. Data are expressed as mean ± SD (n = 3). Statistical analysis, one-way ANOVA with the wild-type values set as the control for Bonferroni's multiple comparisons test (***$P < 0.001$; ns, not significant).

of the uninformative bidirectional cholesterol exchange between macrophages and plasma), cholesterol efflux from macrophages must greatly exceed cholesterol influx into macrophages throughout the duration of the assays. To upregulate cholesterol efflux, macrophages are treated with cAMP and/or loaded with acLDL (16). However, when the rate of cholesterol efflux from macrophages (cholesterol mass released to plasma per unit of time) is very high, macrophage cholesterol may quickly saturate the cholesterol holding capacity of plasma (cholesterol mass per unit of plasma volume; named after a corresponding metric of bile, see Ref. 17) in the ex vivo cholesterol efflux capacity assay and accumulate in plasma in the in vivo mRCT assay. In this case, cholesterol efflux capacity and the plasma steady-state levels of macrophage-derived radiocholesterol in vivo will estimate plasma cholesterol holding capacity, which is proportional to HDL mass and uninformative about mRCT.

To explore the relationship between PLTP activity and the rate of macrophage cholesterol efflux, the in vivo mRCT assay was modified to measure macrophage-derived radiocholesterol in plasma at very early time points after the assay onset. Early time points reflect the rate of macrophage cholesterol efflux rather than the plasma cholesterol holding capacity. The plasma [^{3}H]cholesterol versus time curves for PLTP deletion and wild-type mice overlaid each other for the first 2 h and diverged only slightly by 4 h after the macrophage injection (Fig. 7A). The curve for PLTP overexpression mice trended lower by 1 h and was significantly lower by the 1.5 h time point, in comparison with the PLTP deletion and wild-type curves. However, steady-state [^{3}H]cholesterol counts and, thus, cholesterol holding capacity of PLTP overexpression plasma were much lower relative not only to wild-type plasma, but also to PLTP deletion plasma. The cholesterol efflux capacity assay was likewise modified to measure cholesterol release from macrophages at early time points. When measured at 30 min (instead of 4 h) after plasma addition to cells, cholesterol efflux from cAMP-treated J774 cells to PLTP overexpression plasma was significantly higher than to wild-type plasma and PLTP deletion plasma; efflux to wild-type and PLTP deletion plasma was the same (A. Picataggi,

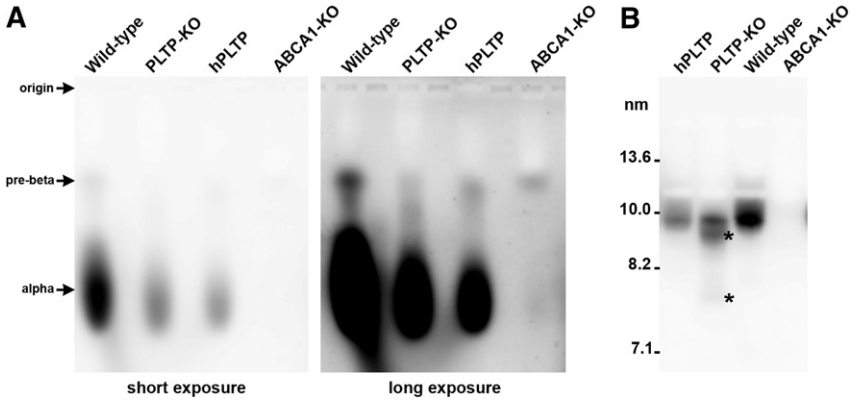

Fig. 6. HDL particle species assortment in plasma from PLTP overexpression and deletion animals. A: Agarose gel electrophoresis analysis of pooled plasma for the levels of preβ HDL. Plasma from ABCA1-null animals (ABCA1-KO) was included as a control to show specificity of the anti-apoA-I antibody and to identify the location of preβ HDL. B: Native polyacrylamide gel electrophoresis analysis of pooled plasma for HDL particle species assortment. Asterisks indicate the two new particle species present in PLTP deletion plasma, but not in wild-type or PLTP overexpression plasma. Representative results.

unpublished observations). These results suggest that PLTP activity does not adversely affect the rate of macrophage cholesterol efflux to plasma, and likely for this reason the in vivo mRCT was not altered in PLTP overexpression and deletion mice.

Even though the rate of macrophage cholesterol efflux to plasma in PLTP overexpression and deletion mice was at or above the wild-type level, for the overall cholesterol transport from macrophages to liver to remain the same, the liver must have a mechanism to ensure that it takes up the same amount of cholesterol regardless of plasma HDL-C concentration. Decreased HDL-C and cholesterol efflux capacity and normal in vivo mRCT have been reported in animals with liver-specific deletion of ABCA1 (10), probucol-mediated suppression of ABCA1 activity (18), whole-body deletion of one LCAT allele (19) and apoE deletion in all tissues except macrophages (20). Increased HDL-C and cholesterol efflux capacity and normal in vivo mRCT have been reported in animals with whole-body deletion of hepatic lipase, endothelial lipase, or both (21). HDL particles are highly heterogenous (12). The liver employs at least three types of receptors to take up HDL: the apoE receptors (22), scavenger receptor class B type I (SR-BI) (23), and the ecto-F_1-ATPase/P_2Y_{13} purinergic receptor pathway (24). The assortment of HDL particles or liver HDL receptors may change to increase or decrease the cumulative affinity between HDL and its receptors to match changes in HDL-C.

PLTP overexpression and PLTP deletion mouse models have been extensively studied. Our findings regarding reduced levels of HDL-C, plasma phospholipid, and apoA-I in PLTP overexpression and deletion mice agree well with the previous reports (3, 4, 25, 26). van Haperen et al. (26) reported that preβ HDL levels are the same in fresh plasma from hPLTP transgenic (hPLTP-tg) and wild-type mice, in contrast to our findings that preβ HDL is lower in PLTP overexpression animals. The difference likely stems from the severity of HDL phenotype: plasma total cholesterol and phospholipid levels were reduced by 38 and 28%, respectively, in hPLTP-tg mice and by 76 and 68%, respectively, in PLTP overexpression animals in the present study. Preβ HDL was lower in PLTP deletion mice in this study, in line with the previous observations (4). Si et al. (27) have reported that PLTP deletion reduces mRCT in mice fed a Paigen-like diet. In our study, mice were fed a chow diet. High-fat high-cholesterol diets raise mRCT three to four times (28). Si et al.'s (27) and our findings together suggest an intriguing possibility that PLTP and plasma factors that are dispensable for mRCT on a regular diet may be required for robust mRCT on a high-fat high-cholesterol diet.

Samyn et al. (8) reported that hPLTP-tg mice had reduced radiocholesterol counts in plasma, unchanged counts in liver and bile (just as in the present study), and reduced counts in feces (in contrast to our finding of no difference) in comparison with wild-type controls in the in vivo mRCT assay. The authors attributed the low fecal counts to increased expression of PLTP in the intestine, where it promotes cholesterol absorption (29). In the present study, *PLTP* was expressed using a hepatocyte-specific thyroxine binding globulin promoter, which is not active in other liver cells, such as Kupffer cells, or other organs (30, 31). Because of this, PLTP levels were normal in the intestine of PLTP overexpression mice, unlike in hPLTP-tg animals. However, a decrease in intestinal cholesterol absorption in PLTP deletion animals could have increased fecal counts. This was not observed. The effect of PLTP deletion on cholesterol absorption is mild and may not be detectable unless cholesterol is provided as a bolus (29). Mouse studies have shown that plasma and tissue PLTP activities are not correlated (32). In human studies, PLTP activity has been measured in plasma (33–35). The usage of the thyroxine binding globulin promoter allowed us to increase plasma PLTP through hepatocyte-specific expression without overexpressing it in other cell types, thus precluding appearance of other phenotypes and simplifying data interpretation.

The relationship between PLTP activity and HDL mass is also likely to be parabolic in humans. Higher and lower PLTP activity is associated with lower HDL-C in small select

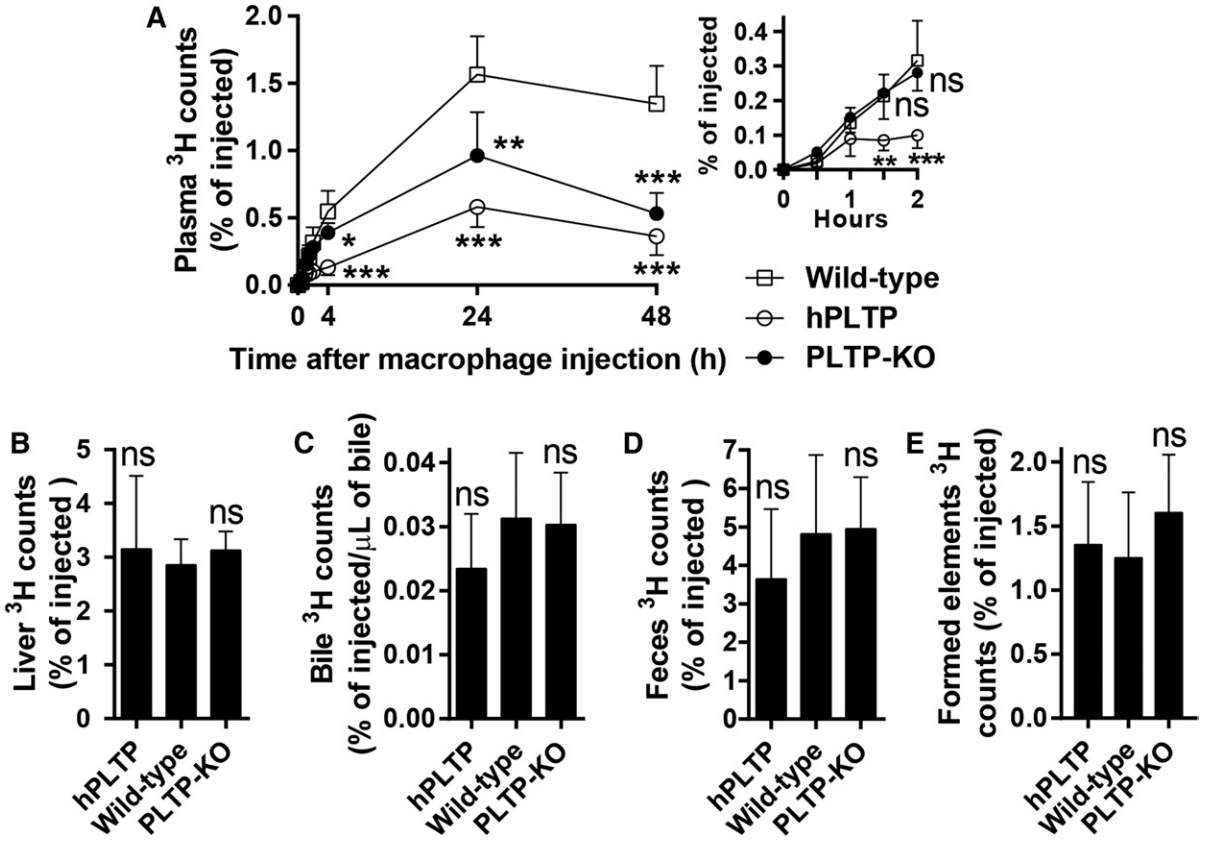

Fig. 7. mRCT in PLTP overexpression and deletion mice. A: After the injection of acLDL-loaded radiocholesterol-labeled J774 cells, blood of wild-type, PLTP overexpression, and PLTP deletion mice was sampled via the tail vein at the indicated time points, except for the 48 h sampling, which was conducted via the retro-orbital plexus (the smaller panel in the upper-right corner shows the early blood sampling time points in greater detail). Data are expressed as mean ± SD. Statistical analysis, one-way ANOVA by time point with Bonferroni's multiple comparisons test to compare wild-type values with hPLTP and PLTP-KO values (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, not significant). B–D: The [^{3}H]cholesterol counts in liver, bile, and feces from wild-type, PLTP overexpression, and PLTP deletion mice. E: The 48 h formed elements fraction (i.e., blood cells pelleted by centrifugation away from plasma) was solubilized and read for [^{3}H]cholesterol, and the counts were expressed as a percent of the total counts injected with cells. Data are expressed as mean ± SD. Statistical analysis, one-way ANOVA with the wild-type values set as the control for Bonferroni's multiple comparisons test (ns, not significant).

human populations (36–38). A large study did not find an association between PLTP activity and HDL-C, possibly because a positive and negative correlation between the two offset each other (39). No *PLTP*-deficient individuals have been identified to the best of our knowledge. However, PLTP activity is significantly reduced in subjects with loss of function in UDP-GalNAc:polypeptide α-*N*-acetylgalactosaminyltransferase T2 (GALNT2), an enzyme that glycosylates PLTP (40). These subjects have reduced HDL-C levels. Both direct and inverse association between PLTP activity and atherosclerotic CVD (ASCVD) has been reported in human studies (33–35, 39). It is well-established that higher PLTP activity promotes atherosclerosis in mouse models (1). Whole-body deletion of PLTP in mice has been reported to reduce atherosclerosis (41), but deletion of macrophage PLTP has been shown to increase atherosclerosis in both apoE and LDL receptor deletion backgrounds (42, 43). The relationship between PLTP and ASCVD may be parabolic, with both high and low levels of PLTP activity promoting atherosclerosis by different mechanisms. As mentioned above, a recent study (27) showed that PLTP is required for mRCT on a high-fat high-cholesterol diet. This finding suggests an intriguing new hypothesis for the role of PLTP in mRCT and ASCVD: PLTP activity that is lagging or leading the optimal level impairs mRCT and accelerates ASCVD in diet-induced hyperlipidemia.

The present study has several limitations. In the cholesterol efflux capacity assay, J774 cells are treated with cAMP to upregulate ABCA1, while in the in vivo mRCT assay, the cells are not treated with cAMP, but are loaded with acLDL. acLDL loading does not induce ABCA1 expression as much as cAMP treatment (E. Cipollari, unpublished observations; Ref. 16). Therefore, the cholesterol efflux capacity assay likely overestimates ABCA1-dependent efflux, while the in vivo mRCT assay may underestimate it. Incubation of PLTP overexpression plasma for 3 h at 37°C has been shown to increase preβ HDL above the amount present in fresh plasma (26). Dilution of plasma with cell medium 50–100 times in the cholesterol efflux capacity assay likely tempers the rate of HDL modification by reducing concentrations of HDL particles and plasma factors, but does not eliminate it. A comparison of reported values for PLTP activity and HDL-C across several studies suggests that absolute and relative values of PLTP activity vary widely and are not correlated well with HDL-C (e.g., Refs. 26, 33). Finally, mouse plasma lipid metabolism and mRCT reflect the

corresponding processes in humans only partly, and thus mouse findings may not fully apply to human physiology.

In summary, PLTP overexpression and deletion reduce HDL mass and plasma cholesterol holding capacity without adversely affecting the rate of cholesterol efflux from macrophages to plasma and macrophage cholesterol transport in plasma to liver. These findings imply a substantial resilience of mRCT in the face of drastic changes in HDL metabolism. We integrate our finding with previously published reports and further advance the hypothesis that an optimum PLTP activity is required to maintain mRCT when plasma lipid levels are elevated owing to consumption of high-fat high-cholesterol diet and that failure to keep PLTP activity at the optimum in hyperlipidemia promotes atherosclerosis.

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