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Plasma Cholesteryl Ester Transfer Protein Is Predominantly Derived From Kupffer Cells

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The role of Kupffer cells (KCs) in the pathophysiology of the liver has been firmly established. Nevertheless, KCs have been underexplored as a target for diagnosis and treatment of liver diseases owing to the lack of noninvasive diagnostic tests. We addressed the hypothesis that cholesteryl ester transfer protein (CETP) is mainly derived from KCs and may predict KC content. Microarray analysis of liver and adipose tissue biopsies, obtained from 93 obese subjects who underwent elective bariatric surgery, showed that expression of *CETP* is markedly higher in liver than adipose tissue. Hepatic expression of *CETP* correlated strongly with that of KC markers, and CETP messenger RNA and protein colocalized specifically with KCs in human liver sections. Hepatic KC content as well as hepatic *CETP* expression correlated strongly with plasma CETP concentration. Mechanistic and intervention studies on the role of KCs in determining the plasma CETP concentration were performed in a transgenic (Tg) mouse model expressing human CETP. Selective elimination of KCs from the liver in CETP Tg mice virtually abolished hepatic CETP expression and largely reduced plasma CETP concentration, consequently improving the lipoprotein profile. Conversely, augmentation of KCs after Bacille-Calemette-Guérin vaccination largely increased hepatic *CETP* expression and plasma CETP. Also, lipid-lowering drugs fenofibrate and niacin reduced liver KC content, accompanied by reduced plasma CETP concentration. *Conclusions*: Plasma CETP is predominantly derived from KCs, and plasma CETP level predicts hepatic KC content in humans. (HEPATOLOGY 2015;62:1710-1722)

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Kupffer cells (KCs) have been identified as the resident macrophages in the liver more than a century ago. KCs have a firmly established function in host defense,[1,2] bilirubin metabolism,[2] and liver regeneration.[3] In addition, KCs play an important role in the pathogenesis of various liver diseases, including liver failure and ischemia-reperfusion injury during liver resection or transplantation, alcohol-induced liver disease, and nonalcoholic fatty liver disease (NAFLD).[4] NAFLD is currently the leading cause of chronic liver diseases in the Western world and the estimated prevalence in the general population ranges between 20% and 30%, rising to as high as 90% in morbidly obese

Abbreviations: ANOVA, analysis of variance; BCG, Bacille-Calmette-Guérin; BMI, body mass index; BMT, bone marrow transplantation; CETP, cholesteryl ester transfer protein; FXR, farnesoid X receptor; HDL, high-density lipoprotein; HDL-C, HDL-cholesterol; IHC, immunohistochemistry; KC, Kupffer cell; LPS, lipopolysaccharide; LXRα, liver X receptor alpha; MARCO, macrophage receptor with collagenous structure; mRNA, messenger RNA; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; TC, total cholesterol; Tg, transgenic; TG, triglyceride; SAT, subcutaneous adipose tissue; SDs, standard deviations; VAT, visceral adipose tissue; VLDL, very-low-density lipoprotein; VLDL-C, VLDL-cholesterol; WT, wild type; WTD, Western-type diet.

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individuals.[5,6] NAFLD embraces a spectrum of liver pathology, from simple steatosis to severe nonalcoholic steatohepatitis (NASH),[7] that is characterized by accumulation of lipid in the liver (steatosis) accompanied by hepatic inflammation (hepatitis). Treatment options for NAFLD are limited.[8]

Experimental studies in mice suggest a pivotal role of KCs in the development of NASH.[9-11] Preliminary data from clinical trials indicate that the number of hepatic KCs correlates with the severity of liver damage in patients with NASH.[12,13] To distinguish NASH from simple steatosis and design more effective and specific treatments for liver injury and inflammation for patients with NASH, it is essential to diagnose KC content in clinical practice. However, liver biopsies are still the gold standard used for this purpose[14] owing to the fact that noninvasive modalities or plasma biomarkers are currently not available to predict KC content. Obviously, liver biopsies have severe limitations, such as sampling error, differences in histopathological interpretation, as well as patient stress and discomfort, risk of bleeding, and long hospitalization. Therefore, noninvasive biomarkers with a high sensitivity and specificity for hepatic KC content are eagerly awaited.

Cholesteryl ester transfer protein (CETP) is a plasma protein that is mainly bound to high-density lipoproteins (HDL) in plasma and plays a pivotal role in metabolism of HDL and very-low-density lipoprotein (VLDL). Currently, CETP inhibition is a target for the treatment of dyslipidemia to ultimately reduce cardiovascular disease risk.[15,16] Previous studies have indicated that liver and adipose tissue are the two major sources of circulating CETP in humans.[17,18] However, the relative contribution of liver and adipose tissue to total plasma CETP and the cell types involved in CETP synthesis remain to be unambiguously determined. Some studies suggested that hepatocytes may be responsible for hepatic expression and secretion of CETP,[17,19] whereas another study suggested that nonparenchymal cells, including KCs, are the principal source of hepatic CETP.[20] Our previous studies indicated that pharmacological treatments that lead to a reduction in KCs are associated with a reduction in plasma CETP.[21] Therefore, in this study, we aimed to determine the cellular origin of CETP in humans. We hypothesized that hepatic CETP expression is confined to KCs, and plasma CETP is predominantly derived from KCs.

Materials and Methods

Design of Human Studies. Two independent populations were selected. The first cohort was obtained from the general population in Rijswijk, The Netherlands, consisting of 1,434 nondiabetic subjects between 40-70 years of age (654 males, 780 females). Exclusion criteria included diagnosed diabetes, known terminal disease, and a history of psychiatric disorder or substance abuse. Waist circumference was measured and venous blood samples were taken after overnight fasting for measurement of plasma CETP concentration. The Rijswijk study was approved by the review board of South West Holland and performed in accord with the Declaration of Helsinki.

The second study consisted of 93 severely obese subjects (body mass index [BMI]: 30-74) who underwent elective bariatric surgery from 2006 to 2009 at the Department of General Surgery, Maastricht University Medical Center (Maastricht, The Netherlands).[22] Subjects using anti-inflammatory drugs or having acute or chronic inflammatory diseases, degenerative diseases, and subjects reporting alcoholic intake >10 g/day were excluded. During surgery, biopsies from liver, subcutaneous adipose tissue, and visceral adipose tissue were taken for messenger RNA (mRNA) isolation and hybridization. Venous blood samples were obtained after

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overnight fasting on the morning of surgery for analysis of the plasma CETP concentration and lipid parameters. Six weeks after bariatric surgery, blood samples were collected for analysis of plasma CETP concentration. This study was approved by the Medical Ethics Board of Maastricht University Medical Center and was in line with the Declaration of Helsinki. All participants provided informed written consent.

Details of all parameters measured in both population cohorts are provided in the Supporting Materials and Methods.

Design of Mouse Studies. Female APOE*3-Leiden.CETP transgenic (Tg) mice expressing the human CETP gene under the control of its natural flanking regions were used[23] and housed under standard conditions with a 12-hour light/dark cycle with free access to food and water, unless indicated otherwise. Mice were fed a chow diet or semisynthetic Western-type diet (WTD), containing 0.1% (w/w) cholesterol, 1% (w/w) corn oil, and 15% (w/w) cocoa butter (Hope Farms, Woerden, The Netherlands).

In a first experiment, mice were fed WTD for 4 weeks, randomized according to body weight and plasma lipid levels (total cholesterol [TC] and triglyceride [TG]), received two intraperitoneal injections of 4 mL/kg body weight liposomal clodronate (20 mg/kg body weight; purchased from Dr. N. van Rooijen, Amsterdam, The Netherlands) at a 3-day interval to deplete macrophages from the liver,[24] and were terminated 3 days after the second injection.

In a second experiment, mice were fed chow diet and randomized according to body weight and plasma lipid levels (TC and TG), received two intravenous injections of Bacille-Calmette-Guérin (BCG) vaccine from the State Serum Institute (SSI; 0.75 mg; 5×10^6 colony-forming units in 100 μL of phosphate-buffered saline; SSI Denmark, Copenhagen, Denmark)[25] at the beginning of the study and after 2 weeks. Mice were terminated 4 weeks after the first injection.

In a third experiment, mice were fed WTD, without (control) and with 0.04% (w/w) fenofibrate or 1% (w/w) niacin (both from Sigma-Aldrich, St. Louis, MO) for 4 additional weeks before sacrificing.

The institutional ethical committee on animal care and experimentation from Leiden University Medical Center (Leiden, The Netherlands) had approved all animal experiments. In all experiments, blood was obtained by tail vein bleeding into heparin-coated capillary tubes after 4 hours of fasting at 12:00 PM with food withdrawn at 8:00 AM. Tubes were placed on ice and centrifuged, and the obtained plasma was snap-frozen in liquid nitrogen and stored at −20°C until further analysis. Plasma was assayed for CETP and lipid concentrations and lipoprotein profiles (see the Supporting Materials and Methods). After mice had been sacrificed, liver and gonadal adipose tissue samples were collected to measure expression of selected genes by quantitative real-time polymerase chain reaction and proteins by immunohistochemistry (see the Supplemental Materials and Methods).

Statistical Analysis. Categorical variables are presented as frequencies and percentages, and continuous variables as means and standard deviations (SDs), or medians and interquartile ranges for variables with skewed distributions. Pearson's correlation was used to estimate the association between waist circumference and plasma CETP in the Rijswijk study. In the bariatric surgery cohort, Spearman's correlation was used to determine the correlation between expression of CETP and macrophage receptor with collagenous structure (MARCO) in the liver, subcutaneous adipose tissue (SAT), and visceral adipose tissue (VAT); the association between CETP expression in liver, SAT, VAT, and plasma CETP level; as well as the association between CETP expression in liver, SAT, VAT, and plasma HDL-cholesterol (HDL-C) level, respectively. For correlation analyses, P values are provided in addition to q values that were calculated after correction for multiple testing. Colocalization between $CETP^+$ cells and $CD68^+$ cells in liver biopsies was determined by Pearson's correlation. For mouse studies, statistical differences between groups were assessed with the Student t test for two independent groups or two-way analysis of variance (ANOVA) with Tukey's post-hoc test for multiple comparisons. All reported P values are two-tailed, and P/q values of less than 0.05 were considered statistically significant.

Results

Waist Circumference Does Not Correlate With the Plasma CETP Level in Humans. Previous studies in a small cohort of 13 men demonstrated a correlation between CETP expression in adipose tissue with plasma CETP level,[26] suggesting that adipose tissue may contribute to the plasma CETP pool. To investigate whether central adiposity correlates with plasma CETP concentration, we first assessed the correlation between waist circumference and plasma CETP concentration in a general population in Rijswijk. The characteristics of 1,434 subjects (654 males and 780 females) are shown in Supporting Table 1. Mean (±SD) waist circumference was 99 ± 11 cm for males and 89 ± 12 cm for females. Median value for plasma CETP concentration was significantly lower in males (2.31 μg/mL [1.90-2.72]), compared to females (2.44 μg/mL [2.02-2.86];

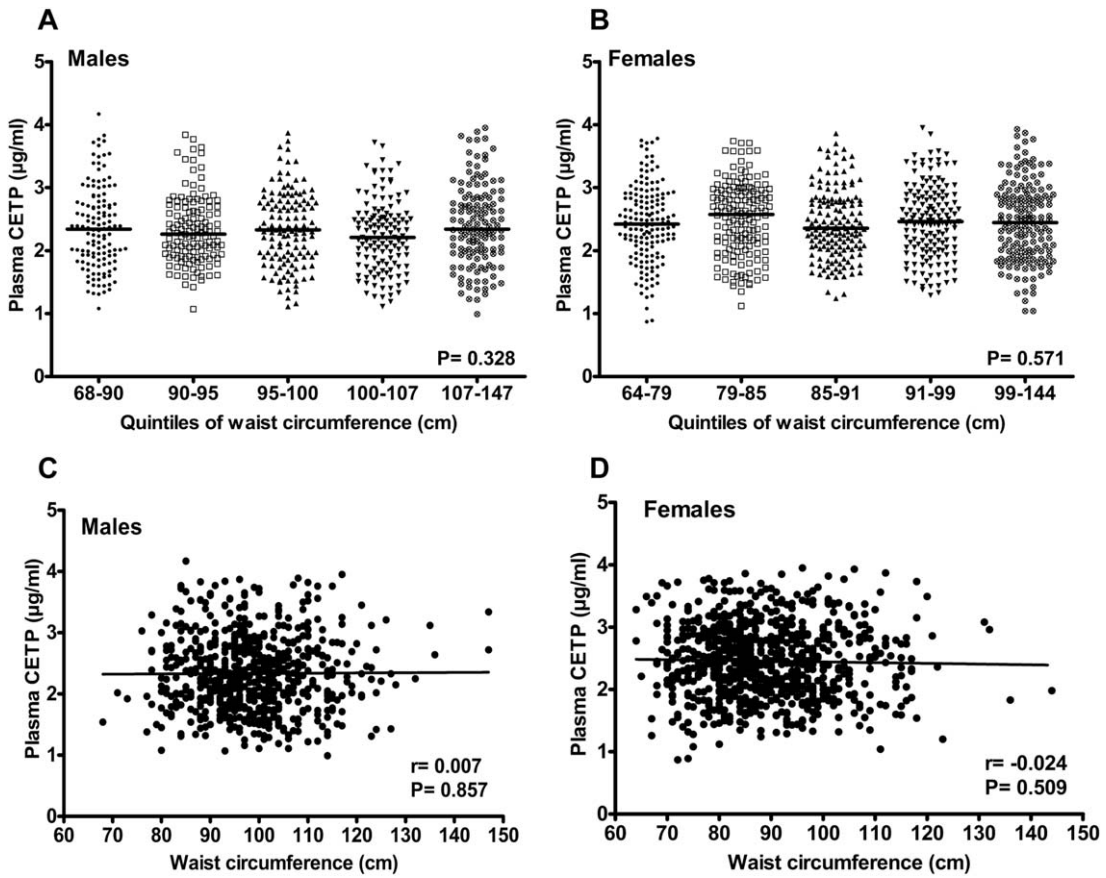

Fig. 1. Waist circumference does not correlate with plasma CETP level in a general population. (A and B) A total of 1,434 subjects were enrolled in the Rijswijk study. Plasma CETP concentration over quintiles of waist circumference in 654 male (A) and 780 female (B) subjects were determined, and medians are indicated. (C and D) Correlations between waist circumference and plasma CETP concentration in male (C) and female (D) subjects.

$P < 0.001$). However, plasma CETP concentration did not differ between quintiles of waist circumference in either males (Fig. 1A) or females (Fig. 1B), and no correlation between waist circumference and plasma CETP concentration was observed in either males (Fig. 1C) or females (Fig. 1D). Plasma CETP level does not correlate with plasma HDL-C level in either males ($r = -0.015$) or females ($r = 0.011$). Exclusion of subjects who received statins (82 males and 61 females), known to reduce plasma CETP level,[27] did not change the association between waist circumference and plasma CETP concentration for males (Supporting Fig. 1A) and females (Supporting Fig. 1B). Thus, these findings suggest that adipose tissue mass and plasma CETP are not correlated in humans.

Hepatic CETP Expression Is Exclusive to KCs and Correlates With the Plasma CETP Level in Humans. We next compared expression of *CETP* in biopsies from livers, SAT, and VAT of 93 subjects who underwent elective bariatric surgery, on which microarray analyses have previously been performed.[22] The *CETP* mRNA transcript appeared to be much more abundant in liver than in SAT and VAT (Fig. 2A). Given that the liver contains multiple cell types, we set out to evaluate the cell type(s) responsible for hepatic *CETP* expression. First, we evaluated the correlation between hepatic expression of *CETP* with hepatic expression of the other genes determined by microarray. *CETP* showed the highest correlation with the macrophage-specific marker, *MARCO* ($r = 0.590$; $P = 9.78 \times 10^{-9}$; $q = 8.89 \times 10^{-5}$; Fig. 2B), which is exclusively expressed in KCs.[28] *MARCO* expression in liver is higher than in SAT and VAT (Fig. 2A). Using a publicly available, considerably larger data set of subjects undergoing bariatric surgery ($n = 1{,}008$),[29] we were able to replicate the strong association between *CETP* expression and *MARCO* in liver (Spearman's correlation: $r = 0.624$; $p = 1.74 \times 10^{-71}$; $q = 1.22 \times 10^{-68}$; Supporting Fig. 3). In addition to MARCO, expression of hepatic CETP also correlates with other macrophage/KC markers, for example, CD68 ($r = 0.474$, $P = 9.20 \times 10^{-6}$; $q = 0.03$), CD163 ($r = 0.430$; $P = 6.62 \times 10^{-5}$; $q = 0.096$), and VSIG4

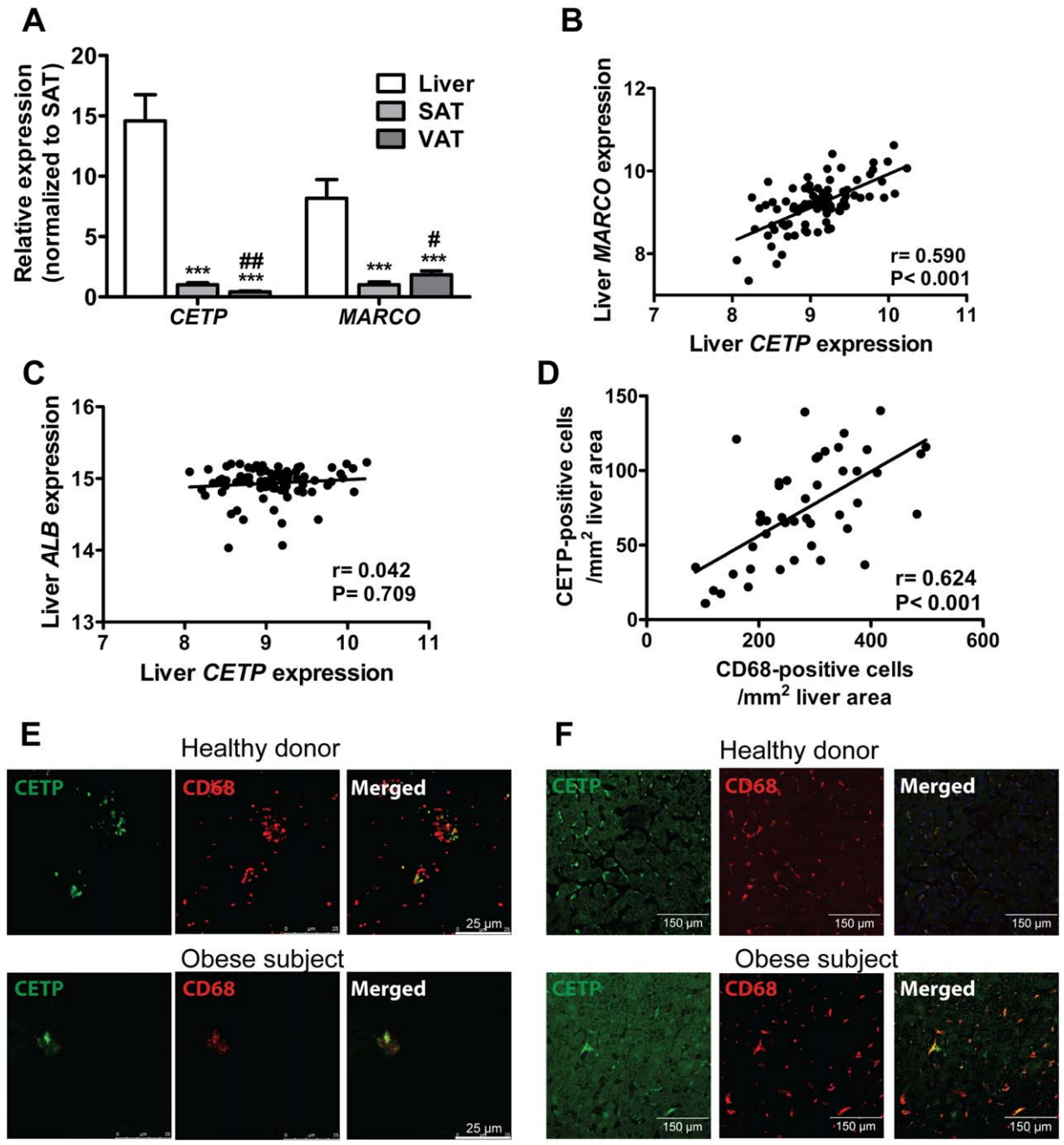

Fig. 2. KCs are the principal source of CETP in the human liver. (A) Biopsies from liver, SAT, and VAT were taken during bariatric surgery from 93 patients and assayed for genome-wide gene expression profiles. *CETP* and *MARCO* expression in liver and VAT were normalized to expression in SAT, respectively. Data are represented as mean ± SD. Two-way ANOVA, $***P < 0.001$, as compared to liver; $\#P < 0.05$, $\#\#P < 0.01$, as compared to SAT. (B) Correlation of expression of *CETP* with *MARCO* in liver. (C) Correlation of expression of *CETP* with *ALB* in liver. (D) Correlation of number of $CETP^+$ cells with $CD68^+$ cells in liver. (E) Representative pictures of *in situ* hybridization of *CETP* mRNA (green), *CD68* mRNA (red), and merged in liver sections both from a healthy donor and an obese subject. (F) Representative pictures of immunofluorescent staining of CETP (green), CD68 (red), and merged in liver sections both from a healthy donor and an obese subject.

(r = 0.408; $P = 1.62 \times 10^{-4}$; $q = 0.147$). In contrast, the expression of hepatic CETP does not correlate with hepatocyte/parenchymal cell–specific markers, for example, ALB (r = 0.042; $P = 0.709$; Fig. 2C), ASGR1 (r = −0.012; $P = 0.908$), and APOA1 (r = 0.238; $P = 0.832$).

To evaluate whether KCs are the primary site of CETP expression and synthesis, we next performed *in situ* hybridization and immunohistochemistry (IHC) on liver sections from a healthy donor and subjects of the bariatric surgery cohort. Indeed, *CETP* mRNA could only be detected in cells that also express *CD68* (Fig. 2E), and

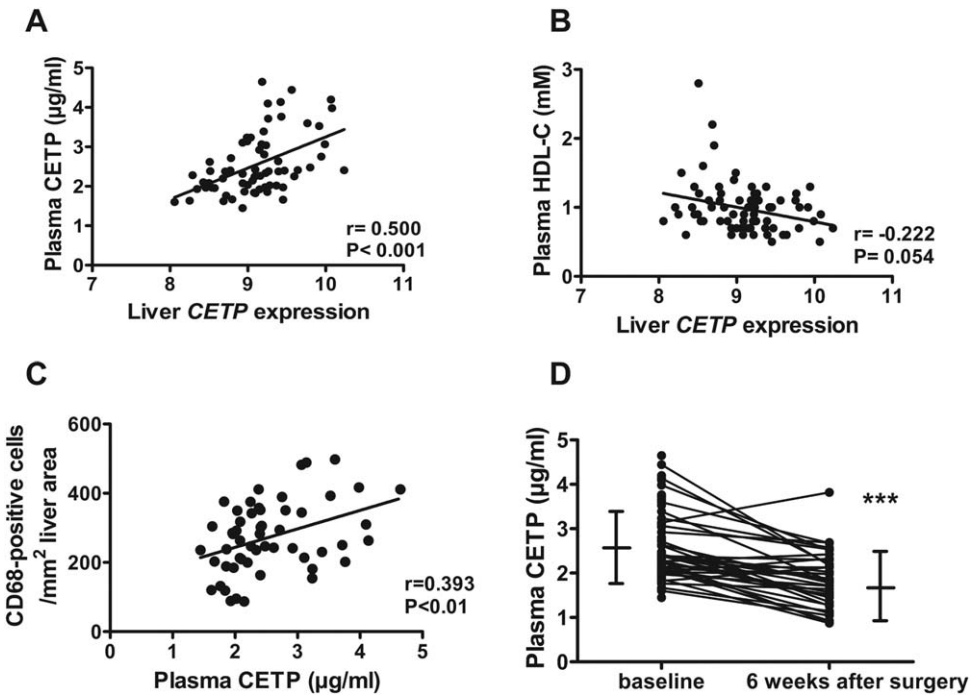

Fig. 3. CETP expression in KCs correlates with plasma CETP and HDL-C in humans. Biopsies from liver, SAT, and VAT were taken during bariatric surgery from 93 patients and assayed for genome-wide gene expression profiles. (A) Correlation of hepatic *CETP* expression and plasma CETP level. (B) Correlation of hepatic *CETP* expression and plasma HDL-C level. (C) After IHC staining of CD68 in liver biopsies, $CD68^+$ KC number were quantified and correlated with plasma CETP level. (D) Before and 6 weeks after bariatric surgery, plasma CETP concentration was measured. All data are represented as mean ± SD; n = 42-68; Student paired *t* test; $***P < 0.001$, as compared to baseline.

CETP protein colocalized with CD68 protein (Fig. 2F). The number of CETP-expressing cells was significantly correlated with the number of $CD68^+$ cells ($r = 0.624$; $P < 0.001$; Fig. 2D), with 39.0% colocalization (slides were available of 46 patients; two to four slides were examined per patient). A similar percentage of colocalization was observed in a healthy subject (38.5%). These data confirm that CETP is expressed and synthesized by KCs in the liver.

Importantly, hepatic *CETP* expression positively correlated with plasma CETP concentration ($r = 0.500$; $P < 0.001$; Fig. 3A) and tended to inversely correlate with the plasma HDL-C level ($r = -0.222$; $P = 0.054$; Fig. 3B). These data suggest that hepatic CETP expression by KCs determines plasma CETP level as well as the CETP-induced effects on plasma HDL-C. A significant correlation was found between the number of $CD68^+$ KCs and plasma CETP concentration ($r = 0.393$; $P < 0.01$; Fig. 3C). This correlation is independent of other clinical parameters, such as gender, BMI, and age. In addition, 6 weeks after bariatric surgery, plasma CETP concentration was significantly decreased, as compared to baseline ($P < 0.001$; Fig. 3D).

In addition to the liver, adipose tissue also contains a large population of resident macrophages. However, *CETP* expression did not correlate with *MARCO* in either SAT (Supporting Figs. 2A and 3) or VAT (Supporting Figs. 2B and 3) in both cohorts of subjects who underwent bariatric surgery.

Elimination of KCs Abolishes Hepatic CETP Expression and Largely Reduces Plasma CETP Concentration in Human CETP Tg Mice. To further investigate the contribution of *CETP* expression in KCs to the plasma CETP level and lipoprotein metabolism, detailed mechanistic studies were performed using APOE*3-Leiden.CETP Tg (E3L.CETP) mice, a well-established mouse model for human-like lipoprotein metabolism that express the human *CETP* gene under control of its natural regulatory flanking regions.[30] *CETP* expression in liver was much higher than in adipose tissue, peritoneal macrophages, and bone marrow–derived macrophages (Supporting Fig. 4A). The higher ratio between CETP expression and F4/80 expression in liver versus adipose tissue suggests that the capacity of KCs to produce CETP is much higher than that of macrophages in adipose tissue (Supporting Fig. 4B). Moreover, both *CETP* mRNA (Supporting Fig. 4B) and protein (Supporting Fig. 4C) specifically colocalized with $F4/80^+$ KCs in liver of mice. In E3L.CETP mice fed a chow diet, $55.9 \pm 13.3\%$ $F4/80^+$ KCs expressed CETP, and the WTD did not affect colocalization percentage ($57.4 \pm 12.4\%$). These data suggest that

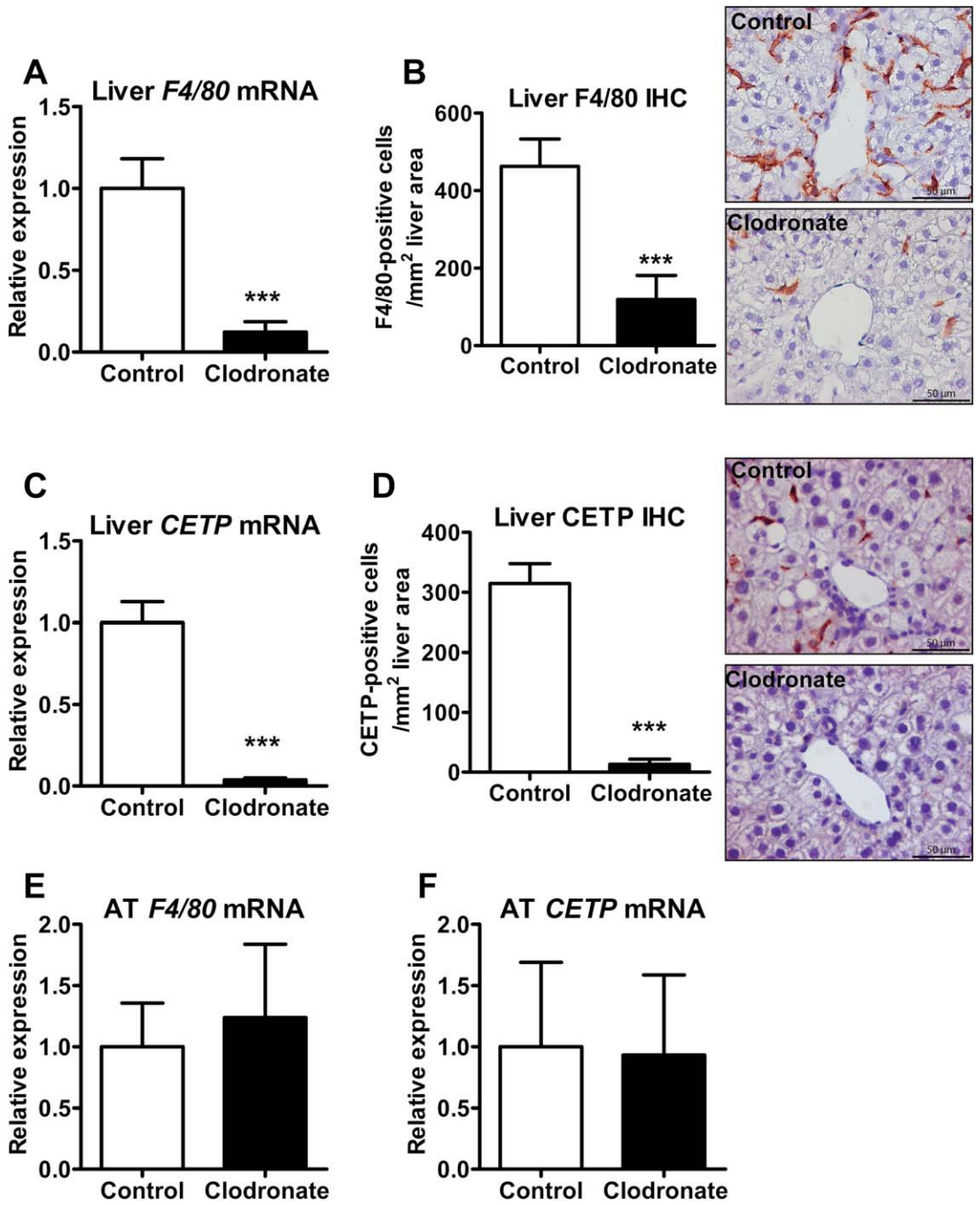

Fig. 4. Elimination of KCs abolishes hepatic CETP expression in human CETP Tg mice. (A) APOE*3-Leiden.CETP mice fed a WTD were treated with or without liposomal clodronate and livers were assayed for *F4/80* mRNA expression. (B) KC content ($F4/80^+$ cells) of the liver. Right panel shows representative pictures of IHC staining of F4/80 in liver sections of each group. (C) mRNA expression of *CETP* in liver. (D) Quantification of hepatic content of $CETP^+$ cells. Right panel shows representative pictures of IHC staining of CETP in liver sections of each group. (E and F) Expression of *F4/80* mRNA (E) and *CETP* mRNA (F) in gonadal adipose tissue. All data are represented as mean ± SD; n = 6; Student unpaired *t* test, $***P < 0.001$, as compared to control group.

E3L.CETP Tg mice have a similar CETP expression pattern as humans.

Next, mice fed a WTD were injected with liposomal clodronate, a well-established method to deplete macrophages from liver in rodents.[24] Indeed, as compared to controls, liposomal clodronate markedly reduced hepatic *F4/80* expression (−88%; Fig. 4A) and the number of $F4/80^+$ KCs (−75%; Fig. 4B). Depletion of KCs almost completely abolished hepatic *CETP* expression (−96%; Fig. 4C) as well as the number of $CETP^+$ cells (−95%; Fig. 4D). Liposomal clodronate did not influence expression of other hepatic immune cell markers, including

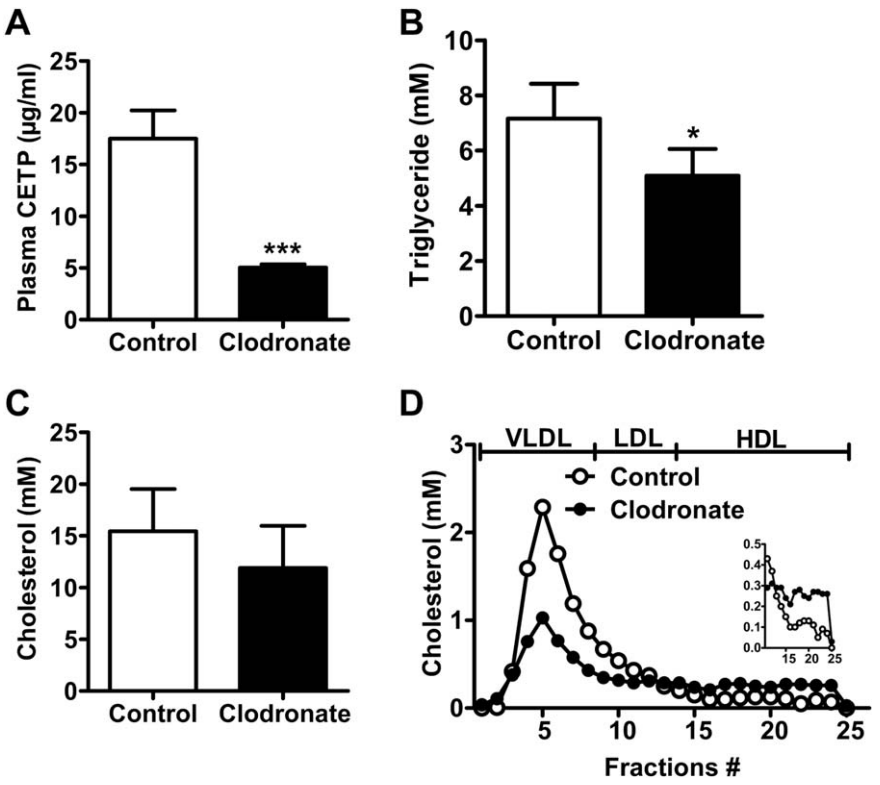

Fig. 5. Elimination of KCs largely reduces plasma CETP level in human CETP Tg mice. APOE*3-Leiden.CETP mice fed a WTD were treated with or without liposomal clodronate. Plasma level of (A) CETP, (B) TGs, and (C) total cholesterol was determined. All data are represented as mean ± SD; n = 6; Student unpaired *t* test: $*P < 0.05$, $***P < 0.001$, as compared to control group. (D) Plasma cholesterol distribution over lipoproteins was assayed by FPLC, with HDL-C fraction as shown in the insert. Abbreviations: FPLC, fast protein liquid chromatography; LDL, low-density lipoprotein.

those of monocytes (CD11b), T cells (CD3, CD4, and CD8), and B cells (CD19 and CD20). In contrast to the liver, liposomal clodronate did not alter mRNA expression of *F4/80* (Fig. 4E) or *CETP* (Fig. 4F) in adipose tissue. Elimination of KCs largely reduced plasma CETP concentration (Fig. 5A) and improved dyslipidemia with respect to decreasing plasma TGs (Fig. 5B), and tended to decrease plasma total cholesterol (Fig. 5C). Lipoprotein profiling showed a reduction in VLDL-cholesterol (VLDL-C; Fig. 5D) and increase in HDL-C (Fig. 5D). Liposomal clodronate had essentially the same effects in mice fed a regular chow diet with respect to decreasing hepatic *CETP* mRNA, $CETP^+$ cells in liver, and plasma CETP concentration, without affecting *F4/80* mRNA and *CETP* mRNA expression by adipose tissue (Supporting Fig. 5).

Augmentation of KCs Largely Increases Hepatic CETP Expression and Plasma CETP Concentration in Human CETP Tg Mice. To investigate whether an increase of KCs by a nondietary trigger increases plasma CETP concentration, E3L.CETP mice fed a regular chow diet were treated with BCG vaccine. As compared to controls, BCG vaccination did not affect food intake or body weight, whereas it largely increased hepatic *F4/80* expression (5.6-fold; Fig. 6A), concomitant with increased hepatic *CETP* expression (2.9-fold; Fig. 6B) and increased plasma CETP concentration (3.3-fold; Fig. 6C). This was accompanied by increased plasma total cholesterol (Fig. 6E), specifically within VLDL (Fig. 6F).

Collectively, we showed, by elimination or augmentation of hepatic KC content, that KCs are the principal source of the plasma CETP pool and that *CETP* expression in KCs determines plasma cholesterol distribution, which is in full accord with the data of our human studies.

Lipid-Lowering Agents Reduce Plasma CETP Level Accompanied by Reduced KC Content in Human CETP Tg Mice. Fibrates and niacin, classical lipid-lowering drugs used for the treatment of dyslipidemia,[31,32] both increase plasma HDL-C in addition to decreasing plasma (V)LDL-C and TGs. This is, at least partly, explained by a reduction in plasma CETP concentration.[33,34] Based on our present data that CETP is largely derived from KCs, we studied whether these lipid-lowering drugs lower hepatic KC content. In line with our previous findings,[33,34] treatment of E3L.CETP mice with fenofibrate or niacin for 3 weeks markedly decreased plasma CETP level (Fig. 7A) and hepatic expression of *CETP* (Fig. 7B). Both drugs also lowered plasma TGs (Fig. 7C) and cholesterol (Fig. 7D), explained by a reduction in VLDL-TGs (Fig. 7E) and VLDL-C (Fig. 7F), and an increase in HDL-C (Fig. 7F). Fenofibrate and niacin decreased liver KC content,

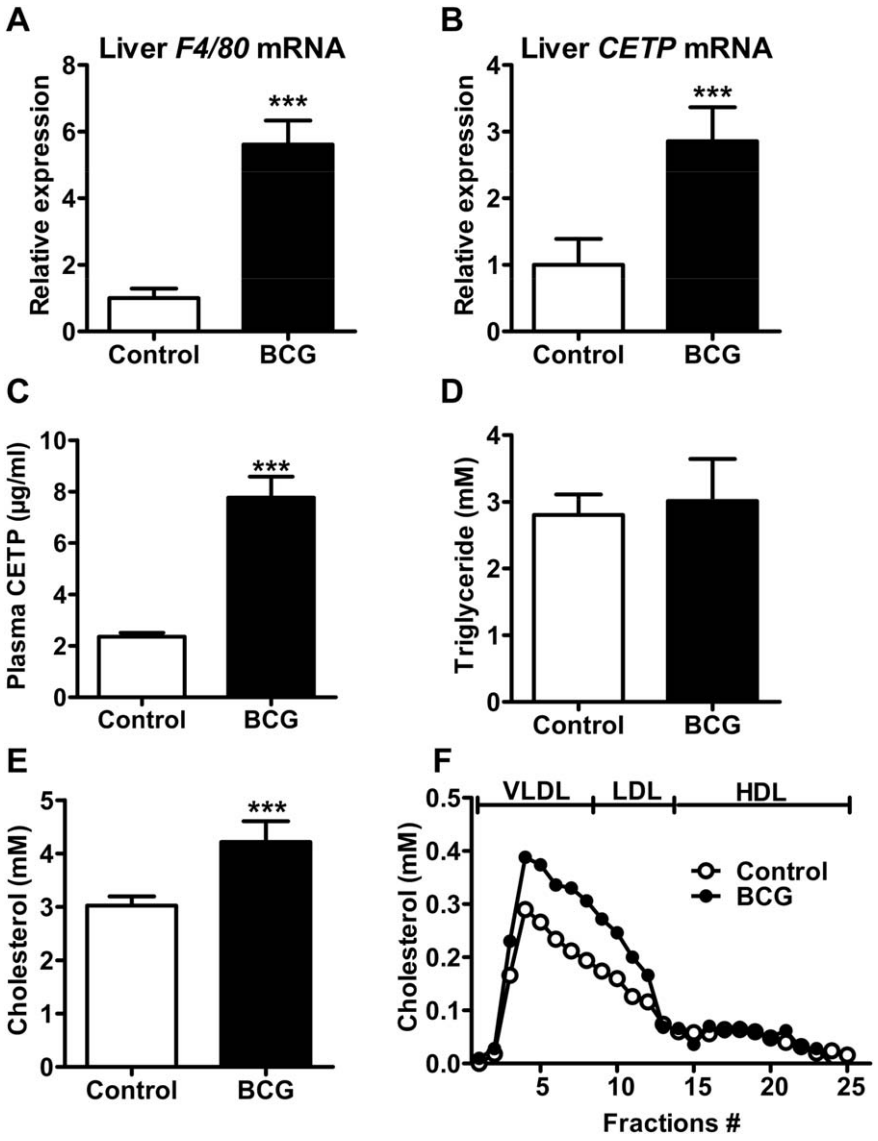

Fig. 6. Augmentation of KCs increases hepatic CETP expression and plasma CETP concentration in human CETP Tg mice. APOE*3-Leiden.CETP mice fed a regular chow diet were treated with BCG vaccine (t = 0 and 2 weeks) and terminated after 4 weeks. Hepatic mRNA expression of *F4/80* (A) and *CETP* (B) were determined. Plasma level of CETP (C), TGs (D), and total cholesterol (E) was determined. All data are represented as mean ± SD; n = 6; Student unpaired *t* test: $***P < 0.001$, as compared to control group. (F) Plasma cholesterol distribution over lipoproteins was assayed by FPLC. Abbreviations: Con, control; Fen, fenofibrate; FPLC, fast protein liquid chromatography; Nia, niacin.

reflected by a reduction in hepatic *F4/80* expression (Fig. 8A), as well as the number of $F4/80^+$ KCs (Fig. 8B,C). Moreover, plasma CETP level strongly correlates to liver F4/80-positive KC content ($r = 0.633$; $P < 0.001$; Fig. 8D). We cannot rule out that lipid-lowering agents reduce KC content as a consequence of improving lipoprotein profile. However, our data are consistent with the hypothesis that fenofibrate and niacin reduce liver KC content, thereby decreasing hepatic CETP production and plasma CETP level, concomitantly improving plasma lipoprotein cholesterol distribution.

Discussion

In this study, we show that the liver is the main source of plasma CETP, and that KCs are responsible for hepatic expression of *CETP* in humans.

Previous studies have shown that *CETP* mRNA is not only expressed in the liver, but also in adipose tissue in several mammalian species.[18] A small human cohort study also found a correlation between adipose tissue *CETP* expression and plasma CETP concentration.[26] We now show that *CETP* is expressed more prominently in the liver, as compared to adipose tissue. In addition, we found no association between waist circumference and plasma CETP level in a large cohort study. Furthermore, by analyzing liver biopsies from patients undergoing bariatric surgery, we found that plasma CETP level strongly correlates with *CETP* expression in the liver, but not in adipose tissue, indicating that *CETP* expression in liver contributes largely to the total plasma CETP pool in humans. Previous studies have shown that lowering adiposity induced by body-weight reduction reduced *CETP* expression and improved lipoprotein metabolism, implying that a reduction in adipose

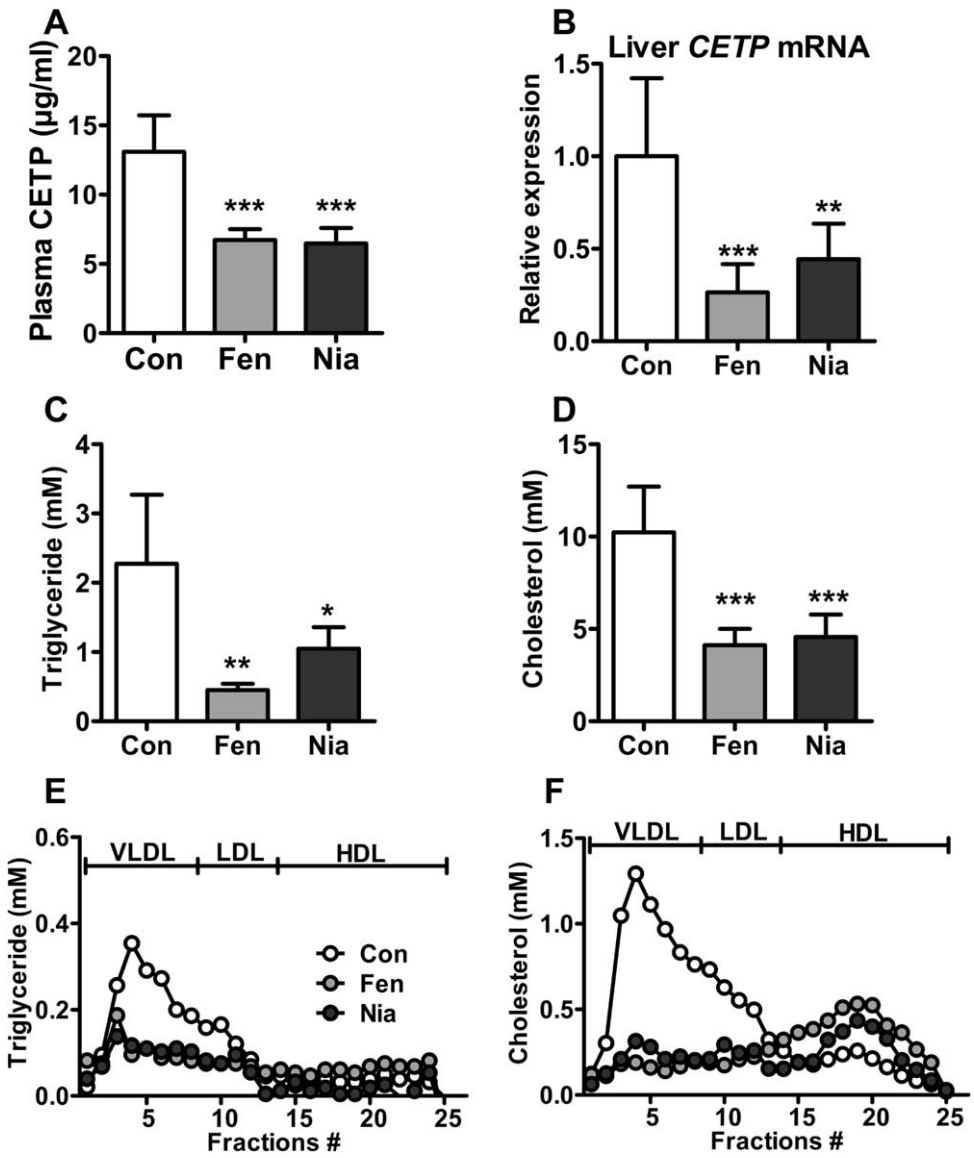

Fig. 7. Lipid-lowering agents reduce plasma CETP level in human CETP Tg mice. APOE*3-Leiden.CETP mice fed a WTD were treated without (Con) or with fenofibrate (Fen) or niacin (Nia) for 4 weeks and plasma was assayed for CETP level (A), TG (C), and total cholesterol level (D), and hepatic mRNA expression of *CETP* (B) were determined. All data are represented as mean ± SD; n = 8; two-way ANOVA: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, as compared to control (Con) group. Distribution of triglyceride (E) and cholesterol (F) over plasma lipoproteins was assayed by FPLC. Abbreviation: FPLC, fast protein liquid chromatography.

tissue reduces plasma CETP.[35,36] However, in addition to reducing adiposity, body-weight reduction also significantly attenuates hepatosteatosis.[37,38] Because we recently demonstrated that a decrease in hepatic lipid content is accompanied by a decrease in plasma CETP level,[38] it is thus tempting to speculate that body-weight reduction by attenuation of hepatosteatosis reduces production of CETP by the liver. Indeed, we observed that plasma CETP level was largely decreased after bariatric surgery. Thus, reduction in plasma CETP level is fully in line with the reduction in liver steatosis and inflammation after bariatric surgery.

Owing to the fact that the liver consists of multiple cell types, we set out to evaluate the cell type responsible for expression of *CETP*. In the present study, we found that expression of established macrophage markers strongly associates with expression of hepatic *CETP*, and that CETP is specifically colocalized with $CD68^+$ KCs in the liver. Hepatic $CD68^+$ cell number and *CETP* expression did not differ among the various liver lobular inflammation scores,[39] indicating no association between the inflammatory foci number and KC content or CETP expression. However, our results clearly indicated that plasma CETP and the number of $CD68^+$ KCs was correlated. Mechanistic studies in E3L.CETP mice showed that depleting KCs from the liver by clodronate liposomes virtually abolished hepatic *CETP* expression and largely reduced plasma CETP level. On the other hand, BCG vaccination markedly increased KCs, as reflected by increased hepatic *F4/80* expression, accompanied by marked increased in hepatic *CETP* expression and increased plasma CETP level. These data fully corroborate

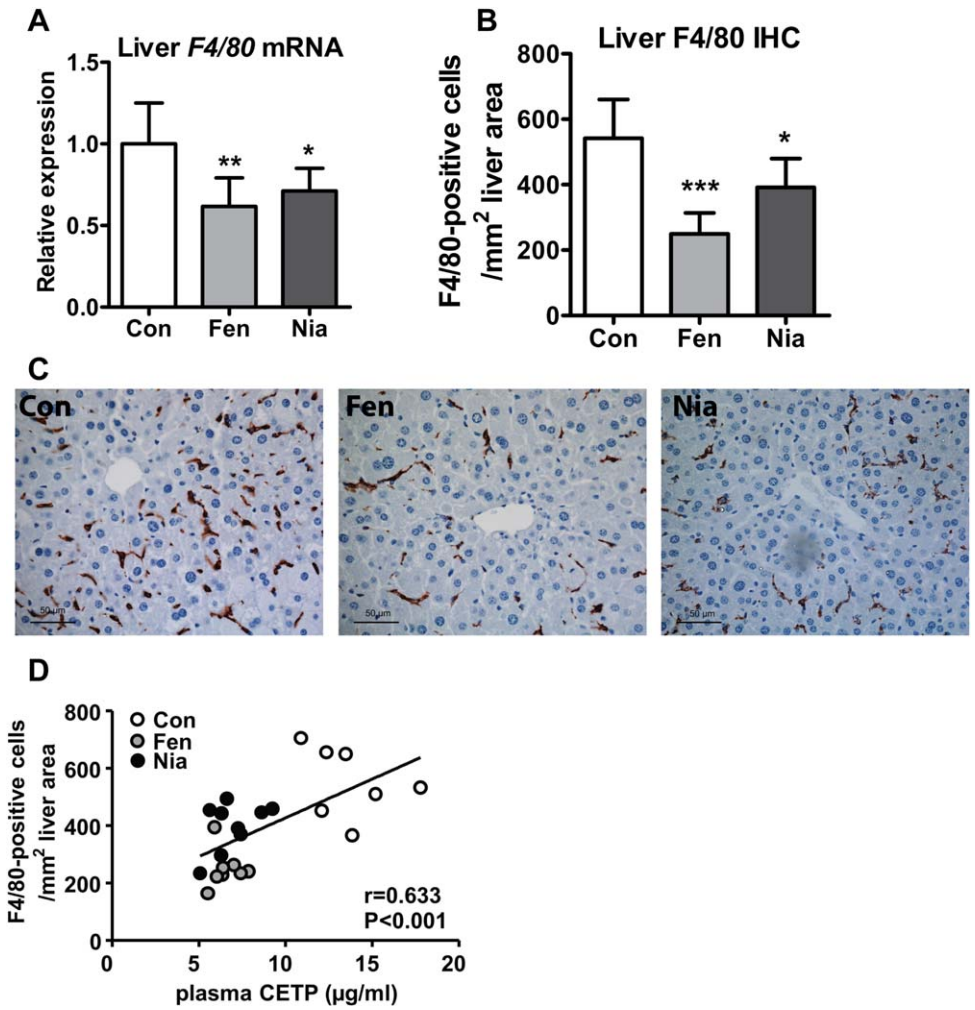

Fig. 8. Lipid-lowering agents reduce the Kupffer cell content in human CETP transgenic mice. APOE*3-Leiden.CETP mice fed a WTD diet were treated without (Con) or with fenofibrate (Fen) or niacin (Nia) for 4 weeks. Hepatic mRNA expression of *F4/80* (A) and KC content ($F4/80^+$ cells; B) were determined. All data are represented as mean ± SD; n = 8; two-way ANOVA: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, as compared to control (Con) group. (C) Representative pictures of IHC staining of F4/80 in liver sections of each group. (D) Correlation of plasma CETP level with KC content ($F4/80^+$ cells).

our findings in humans that KCs, rather than hepatocytes, are the main cellular source of hepatic CETP expression and the plasma CETP pool. In contrast to KCs, we could hardly detect any *CETP* expression in extrahepatic macrophages, including peritoneal macrophages, bone marrow–derived macrophages, and macrophages in adipose tissue (Supporting Fig. 4A,B). It has been reported that CETP expression is regulated by the activation of liver X receptor alpha (LXRα),[40] which is highly expressed in multiple organs. Recently, Gautier et al.[41] demonstrated that, in addition to an LXRα-responsive element in the *CETP* promotor, the *CETP* gene contains an ER8 farnesoid X receptor (FXR) response element in the first intron. Therefore, bile acids that are the natural ligand for FXR and are produced by hepatocytes may be essential for maintaining high expression of *CETP* in hepatic versus extrahepatic macrophages. In fact, treatment of *E3L.CETP* mice with the bile acid taurocholic acid, greatly increased the hepatic *CETP* transcript as well as plasma CETP level.[41]

Previously, hepatic expression of *CETP* in mice has been attributed to both KCs and hepatocytes, based on studies assessing hepatic *CETP* expression 8 weeks after transplantation of bone marrow from wild-type (WT) littermates into human CETP Tg mice, and vice versa, suggesting that KCs contribute approximately 50% to total hepatic CETP expression.[19] However, it should be realized that the replacement of liver KCs after bone marrow transplantation (BMT) occurs slowly. In the same study, it was found that only 50% of KCs were replaced by donor cells 8 weeks after BMT, accompanied by a 50% reduction in plasma CETP level as well as a 2-fold lower hepatic *CETP* expression in WT → CETP Tg mice, as compared to control transplanted CETP Tg →CETP Tg mice.[19] Interestingly, we found in a prolonged BMT study in which CETP-expressing bone marrow cells were replaced by cells from CETP-deficient mice, hepatic *CETP* expression was approximately −95% lower at 36 weeks after transplantation, compared to the CETP Tg →CETP Tg transplanted

group (Supporting Fig. 6). Although we did not include a CETP Tg →WT transplantation group, liver KCs are likely the predominant source of CETP expression.

In addition, depletion of KCs by liposomal clodronate generates a less atherogenic lipid phenotype (e.g., decreasing TG and increasing HDL-C in E3L.CETP mice). Our findings might shed new light on the development of new strategies for CETP inhibition and treatment of dyslipidemia. Strategies focusing on inhibiting CETP synthesis at its cellular origin may be a promising alternative, to avoid potentially adverse effects of the current CETP inhibitors on the function of HDL.[42,43] We also observed that lipid-lowering drugs, including fenofibrate and niacin, reduce plasma CETP level accompanied by reducing the KC content in E3L.CETP mice. In addition, fenofibrate and niacin improved the lipoprotein profile. The question of whether these pharmacological treatments reduce KCs in response to the improved lipoprotein profile or whether they primarily reduce KCs, which, as a consequence, results in the improved lipoprotein profile, is still obscure and needs to be determined by future research.

In this study, we showed that both a dietary trigger and BCG vaccination increase KC content of the liver, accompanied by increased hepatic CETP expression and plasma CETP level, which suggest that increased plasma CETP may be a marker for increased hepatic KC content. However, inflammatory stimuli, such as lipopolysaccharide (LPS), despite raising KC content, reduce CETP expression per se,[44,45] thereby precluding a rise in plasma CETP level. Thus, although increased plasma CETP level indicates increased KCs, equal or lower plasma CETP level does not necessarily indicate a decrease in KCs in intervention studies modulating such inflammatory stimuli.

We conclude that CETP is mainly produced by KCs, and that plasma CETP level significantly correlates with the number of KCs in humans. Moreover, treatment of CETP Tg mice with niacin and fenofibrate reduces KC content accompanied with a reduction in plasma CETP level. Taken together, these data suggest that measurement of plasma CETP concentration can be developed as a diagnostic and predictive test for liver KC content in clinical practice, which should be tested in large population cohorts. However, it should be noted that use of CETP as a marker for hepatic KC content may be limited under conditions of exposure to LPS or other inflammatory stimuli.

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