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EXENDIN-4 DECREASES LIVER INFLAMMATION AND ATHEROSCLEROSIS DEVELOPMENT SIMULTANEOUSLY BY REDUCING MACROPHAGE INFILTRATION

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ABSTRACT

The etiology of inflammation in the liver and vessel wall, leading to non-alcoholic steatohepatitis (NASH) and atherosclerosis respectively, share common mechanisms including macrophage infiltration. To test both disorders simultaneously, it is highly important to tackle the inflammatory status. Exendin-4, a glucagon-like peptide-1 receptor agonist, reduces hepatic steatosis and has been suggested to reduce atherosclerosis; however its effects on liver inflammation are underexplored. Here, we tested the hypothesis that exendin-4 reduces inflammation in both the liver and vessel wall, and investigated the common underlying mechanism. Female *APOE*3-Leiden.CETP* mice, a model with human-like lipoprotein metabolism that develops human-like atherosclerosis, were fed a cholesterol-containing Western-type diet for 5 weeks and were subsequently treated for 4 weeks with exendin-4 or vehicle. Exendin-4 slightly improved dyslipidemia, but markedly decreased atherosclerotic lesion severity and area (-33%), accompanied with a reduction in monocyte adhesion to the vessel wall (-42%) and macrophage content in the plaque (-44%). Furthermore, exendin-4 reduced hepatic lipid content and inflammation as well as hepatic $CD68^+$ (-18%) and $F4/80^+$ (-25%) macrophage content. This was accompanied by less monocyte recruitment from the circulation as the $Mac\text{-}1^+$ macrophage content was decreased (-36%). Finally, exendin-4 reduced chemokine expression in vivo and suppressed oxidized LDL accumulation in peritoneal macrophages in vitro, dependent on the GLP-1 receptor. In conclusion, exendin-4 reduces inflammation in both the liver and the vessel wall by reducing macrophage recruitment and activation. These data suggest that exendin-4 could be a valuable strategy to treat NASH and arteriosclerosis simultaneously.

INTRODUCTION

Cardiovascular disease (CVD) due to atherosclerosis is the leading cause of morbidity and mortality in the Western world. There is a strong association between atherosclerosis and non-alcoholic fatty liver disease (NAFLD), which raised the interest of the role of the liver in the development of atherosclerosis. NAFLD embraces a pathological spectrum of liver diseases, from steatosis with virtually no evidence of hepatocellular injury or liver inflammation to non-alcoholic steatohepatitis (NASH) and cirrhosis [1]. NASH is characterized by accumulation of fat in the liver in combination with hepatic inflammation [2]. This inflammatory response was assumed to be the consequence rather than the cause of the disease. However, compelling data point to a central initiating role of monocyte recruitment and macrophage activation in the progression of hepatic inflammation in a similar way as in the development of atherosclerosis as we recently reviewed [3]. The potential of a shared etiology between inflammation in the liver and vessel wall leads to putative intervention therapies to tackle both disorders at the same time by targeting macrophage infiltration. This is particularly interesting, since the current standard therapies to reduce inflammation in NASH have only limited effectiveness. Lifestyle intervention, including exercise, is recommended in patients with NASH. However, it appears to be difficult to achieve improvements in NASH in the long run and pharmacological intervention is ultimately required [4].

Glucagon-like peptide-1 receptor (GLP-1R) agonists, such as exendin-4, are currently being validated for the treatment of type 2 diabetes mellitus and have been shown to increase glucose-dependent insulin secretion, to regulate gastric emptying, and to reduce food intake and body weight [5]. In addition to improving glycemic control, GLP-1R activation improves lipid metabolism. We [6] and others [7-9] have shown that GLP-1R activation reduces hepatic steatosis, increases hepatic lipid oxidation, decreases lipogenesis, and attenuates hepatic VLDL production.Collectively, these data indicate the potential of GLP-1R activation to treat NAFLD, but the impact on liver inflammation and atherosclerosis is still uncertain. Moreover, contrasting results have been reported regarding the effects on atherosclerosis in ApoE$^{-/-}$ mice. While Gaspari *et al.* [10] showed clear inhibiting effects on the progression of atherosclerosis, Panjwahi *et al.* [11] did not observe any effects on atherosclerosis development. It should be noted that these mice do not respond to lipid-lowering therapy and lack apoE, a crucial factor for cholesterol efflux from macrophages. Given this controversy, combined with the reduction in hepatic steatosis, it is of significant importance to further explore the therapeutic potential of GLP-1R activation to impact on inflammation in both the liver and vessel wall.

The aim of the current study was to evaluate the effect of exendin-4 treatment on hepatic inflammation in addition to its effect on the development of atherosclerosis, and to elucidate the underlying mechanisms, in A*POE*3-Leiden.CETP* (*E3L.CETP*) mice fed a Western-type diet. We show that exendin-4 reduces the influx of macrophages into both the liver and vessel wall, and thereby limits the progression of hepatic inflammation and atherosclerosis simultaneously.

MATERIALS AND METHODS

Animals

Twelve week old, female *E3L.CETP* transgenic mice expressing human CETP under the control of its natural flanking regions were used [12] and housed under standard conditions in conventional cages with free access to food and water unless indicated otherwise. The animals were fed a semi-synthetic Western-type diet, containing 0.4% (w/w) cholesterol, 1% (w/w) corn oil, and 15% (w/w) cacao butter (Hope Farms, Woerden, The Netherlands) for 5 weeks. After randomization according to body weight, plasma total cholesterol (TC), and triglyceride (TG) levels, an osmotic minipump (model 1004, Alzet DURECT Corp., Cupertino, CA) was implanted subcutaneously in the left back region under light isoflurane anesthesia for the continuous delivery of exendin-4 (50 µg/kg/day, Bachem AG, Bubendorf, Switzerland; dissolved in PBS) or PBS as a control for 4 weeks while the Western-type diet was continued. Experiments were performed after 4 h of fasting at 12:00 pm with food withdrawn at 8:00 am. The Institutional Ethics Committee for Animal Care and Experiments from the Leiden University Medical Center, Leiden, the Netherlands approved all experiments.

Blood sampling, plasma metabolites, and lipoprotein profiles

Blood was obtained via tail vein bleeding into heparin-coated capillary tubes. The tubes were placed on ice and centrifuged, and the obtained plasma was snap-frozen in liquid nitrogen and stored at -20°C until further measurements. Plasma was assayed for glucose (Instruchemie, Delfzijl, The Netherlands) as well as TC, and TG using the commercially available enzymatic kits 236691, 11488872 (Roche Molecular Biochemicals, Indianapolis, IN, USA), respectively. Plasma insulin was measured by ELISA (Mercodia AB, Uppsala, Sweden). The distribution of lipids over plasma lipoproteins was determined using fast protein liquid chromatography. Plasma was pooled per group, and 50 µL of each pool was injected onto a Superose 6 PC 3.2/30 column (Äkta System, Amersham Pharmacia Biotech, Piscataway, NJ, USA) and eluted at a constant flow rate of 50 µL/min in PBS, 1 mM EDTA, pH 7.4. Fractions of 50 µL were collected and assayed for TC as described above.

Plasma CETP concentration

Plasma CETP concentration was measured using the DAIICHI CETP ELISA kit according to manufacturer's instructions (Daiichi, Tokyo, Japan).

Atherosclerosis quantification and monocyte adhesion to the endothelium wall

After 4 weeks of treatment, mice were sacrificed and perfused with ice-cold PBS via the heart. Hearts were isolated and fixed in phosphate-buffered 4% formaldehyde, dehydrated, embedded in paraffin and cross-sectioned (5 µm) through the aortic root area. Cross-sections were stained with hematoxylin-phloxine-saffron to determine lesion area and lesion severity as described before [13, 14]. Briefly, various types of lesions were discerned: no lesions, mild lesions with fatty streak-like lesions containing foam cells, and severe lesions referred to advanced lesions containing foam cells in the media, presence of fibrosis, cholesterol clefts, mineralization and/or necrosis. Additionally, cross-sections were stained with AIA 31420 antiserum (1:3000, Accurate Chemical and Scientific, Westbury, NY, USA) to determine macrophage area and monocyte adhesion to the endothelium wall as described [15]. Lesion area and macrophage area were determined using Leica Qwin-software.

Hepatic lipid content

After 4 weeks of treatment, mice were sacrificed and perfused with ice-cold PBS via the heart, and livers were isolated. Lipids were extracted according to a modified protocol from Bligh and Dyer [16]. Briefly, small liver pieces were homogenized in ice-cold methanol. After centrifugation, lipids were extracted by addition of 1800 µl CH_3OH: $CHCl_3$ (1:3 v/v) to 45 µL homogenate, followed by vigorous vortexing and phase separation by centrifugation (5 min at 2,000 rpm). The $CHCl_3$ phase was dried and dissolved in 2% Triton X-100. TG and TC concentrations were measured as described above. Phospholipids (PL) concentration was measured using a commercial kit (phospholipids B, Wako Chemicals, Neuss, Germany). Liver lipids were expressed as nmol per mg protein, which was determined using the BCA protein assay kit (Pierce, Rockford, IL, USA).

Hepatic gene expression analysis

Total RNA was extracted from liver pieces using the Nucleospin RNAII kit (Macherey-Nagel, Duren, Germany) according to manufacturer's instructions. RNA quality was examined by the lab-on-a-chip method using Experion Std Sens analysis kit (Biorad, Hercules, CA) and RNA concentration was determined by Nanodrop technology (Thermo

Scientific, Wilmington, USA). Total RNA was reverse-transcribed with iScript cDNA synthesis kit (1708891, Bio-Rad), and the obtained cDNA was purified with Nucleospin Extract II kit (636973, Macherey-Nagel, Bioké). Real-time PCR was performed on a CFX96 machine (Bio-Rad), the reaction mixture consisting of SYBR-Green Sensimix (QT615, GC Biotech), cDNA, primers (Biolegio, Nijmegen, The Netherlands; see Supplemental Table 1 for primer sequences), and nuclease-free water in a total reaction volume of 10 µL. mRNA values of each gene were normalized to mRNA levels of cyclophilin (*Cyclo*) and hypoxanthine ribosyltransferase (*Hprt*). Data were calculated as fold difference as compared with the PBS control group.

Supplemental Table 1. Primer sequences used for RT-qPCR

Gene	Forward primer	Reverse Primer
Apoa1	GGAGCTGCAAGGGAGACTGT	TGCGCAGAGAGTCTACGTGTGT
Cd68	ATCCCCACCTGTCTCTCTCA	TTGCATTTCCACAGCAGAAG
CETP	CAGATCAGCCACTTGTCCAT	CAGCTGTGTGTTGATCTGGA
Cyclo	CAAATGCTGGACCAAACACAA	GCCATCCAGCCATTCAGTCT
F4/80	CTTTGGCTATGGGCTTCCAGTC	GCAAGGAGGACAGAGTTTATCGTG
Il-1ß	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
Il-6	TGTGCAATGGCAATTCTGAT	CTCTGAAGGACTCTGGCTTTG
Hprt	TTGCTCGAGATGTCATGAAGGA	AGCAGGTCAGCAAAGAACTTATAG
Mcp-1	AACTGTGTGATTGACAAGCACTTAGAC	TGACAGGATTAATGCAGCAGTGT
Tnfa	AGCCCACGTCGTAGCAAACCAC	TCGGGGCAGCCTTGTCCCTT

Apoa1, apolipoprotein A1; *Cd68*, cluster of differentiation 68; *CETP*, cholesteryl ester transfer protein; *Cyclo*, cyclophilin; *Il-1ß*, interleukin-1ß; *Il-6*, interleukin-6; *Hprt*, hypoxanthine ribosyltransferase; *Mcp-1*, monocyte chemotactic protein-1; *Tnfa*, tumor necrosis factor α.

Liver histology

Paraffin-embedded liver sections were stained for F4/80$^+$ macrophages (1/600; Serotec, Oxford, UK) as described [17]. Frozen liver sections (7 µm) were stained for CD68$^+$ resident macrophages (CD68 marker, FA11) and infiltrated macrophages (macrophage marker, Mac-1) as described previously [15].

LDL isolation, radiolabeling, and oxidation

LDL was isolated from human serum by density-gradient ultracentrifugation as described.[17] LDL was labeled with [^{3}H]cholesteryl oleoyl ether (COEth) by incubation with donor [^{3}H]COEth-containing liposomes in the presence of human lipoprotein-deficient serum. In short, liposomes were created by sonication of 1 mg of egg yolk phosphatidylcholine and 200 µCi of [^{3}H]COEth using a Soniprep 150 (MSE Scientific Instruments, Crawley, UK). Subsequently, LDL was incubated with the liposomes (protein: liposomal phospholipid = 1:8, w/w) for 24 h at 37°C under argon. [^{3}H]COEth-labeled LDL was purified by density gradient ultracentrifugation and dialyzed overnight at 4°C against PBS, pH 7.4. Both LDL and [^{3}H]COEth-LDL were oxidized with 5 µM $CuSO_4$ at 37°C for 20 h. Oxidation was terminated by adding 200 µM EDTA. EDTA and $CuSO_4$ were removed by overnight dialysis at 4°C against PBS, pH 7.4. Proper oxidation of LDL was confirmed by a 2.5-fold increased electrophoretic mobility of oxidized LDL (oxLDL) and [^{3}H]COEth-oxLDL compared to LDL on agarose gel. Protein concentration was determined by the BCA protein assay kit.

Plasma anti-oxLDL antibodies

An EIA/RIA high binding 96-well Costar plate (Corning Inc., Corning, NY, USA) was coated with oxLDL (7.5 µg/mL) in PBS. IgM, IgG1, and IgG2a antibodies against oxLDL in serum were measured using an ELISA Ig detection kit (Zymed Laboratories, San Francisco, CA, USA) according to the manufacturer's protocol.

OxLDL uptake by peritoneal macrophages and Oil-red O staining for foam cells

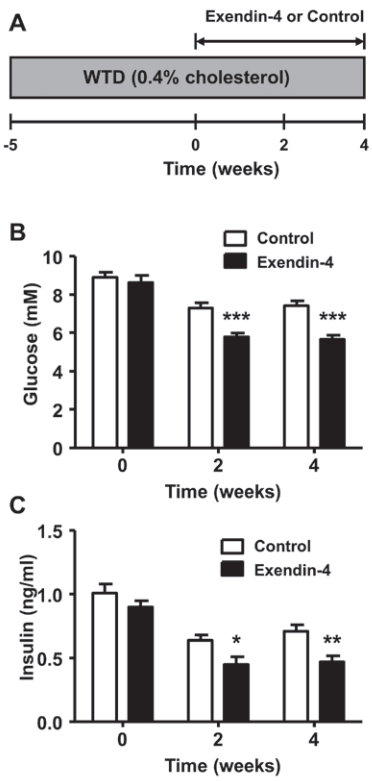
Three days after intraperitoneal injection of 1 mL 4% thioglycollate, peritoneal macrophages from *E3L.CETP* mice were harvested into 10 mL PBS. Subsequently, cells were resuspended in DMEM supplemented with 10% fetal bovine serum, 1% L-glutamine, 100 units/mL penicillin and 100 mg/mL streptomycin, and incubated at 37°C in a humidified 5% CO_2 incubator. Three hours post-plating, cells were washed twice with warm PBS to remove non-adherent cells. Cells were counted and seeded into 24-well plates at a density of 5 x 10^5 cells per well for three days before the experiment. On the experimental day, peritoneal macrophages were washed three times with PBS and incubated in DMEM supplemented with 1% BSA, 100 units/mL penicillin and 100 mg/mL streptomycin for 1 h, followed by DMEM supplemented with 1% BSA, 100 units/mL penicillin, 100 mg/mL streptomycin), 10 µg/mL [^{3}H]COEth-oxLDL, and exendin-4 (0.05 or 0.5 nM) for 48 h at 37°C in a humidified 5% CO_2 incubator. When indicated, exendin-9 (50 nM; Bachem AG, Bubendorf, Switzerland) was added 1 h before addition

of exendin-4. After incubation, macrophages were washed twice with 500 µL PBS and cell lysates were obtained by adding 500 µL of 0.1 M NaOH. 250 µL of cell lysates was used for quantification of ^{3}H-radioactivity. Dpm values were normalized to the total amount of protein (mg) present in 250 µL of cell lysates. Protein concentration in cell lysates was quantified with BCA protein assay kit.

For Oil-red O staining, after incubation, cells were washed twice with PBS and fixed for 30 min in 4% formaldehyde in PBS. Cell were incubated in 60% 2-propanol for 2 min, immediately placed in 60% Oil-red O for 30 min, followed by washing with dH_2O. Cell nuclei were counterstained with hematoxylin for 1 min.

Statistical analysis

All data are presented as means ± SEM. Statistical differences between groups were assessed with the Mann-Whitney *U* test for two independent groups. A *P*-value of less than 0.05 was considered statistically significant.



Supplemental Figure S1. Exendin-4 decreases plasma glucose and insulin levels. After 5 weeks of feeding a Western-type diet containing 0.4% cholesterol, mice were treated with exendin-4 (50 µg/kg/day) or vehicle (PBS) subcutaneously for 4 weeks. Blood was collected by tail bleeding after 4h of fasting before treatment (T=0) and after 2 (T=2) and 4 (T=4) weeks of treatment (A). Plasma glucose (B) and insulin (C) levels were determined. Values are means ± SEM (n=17 mice per group). **P*<0.05, ***P*<0.01, ****P*<0.001 compared to vehicle.

RESULTS

Exendin-4 reduces plasma glucose and insulin levels

E3L.CETP mice were fed a Western-type diet containing 0.4% cholesterol for 5 weeks and thereafter were treated with exendin-4 or PBS via subcutaneous osmotic minipumps for 4 weeks while continuing the diet (Supplemental Fig. S1A). After 2 and 4 weeks of treatment, exendin-4 significantly decreased plasma glucose levels (-21%, $P<0.001$ and -24%, $P<0.001$, respectively, Supplemental Fig. S1B) and insulin levels (-30%, $P<0.05$ and -34%, $P<0.01$, respectively, Supplemental Fig. S1C), which is in line with our previous observations in high-fat diet fed *E3L* mice [6].

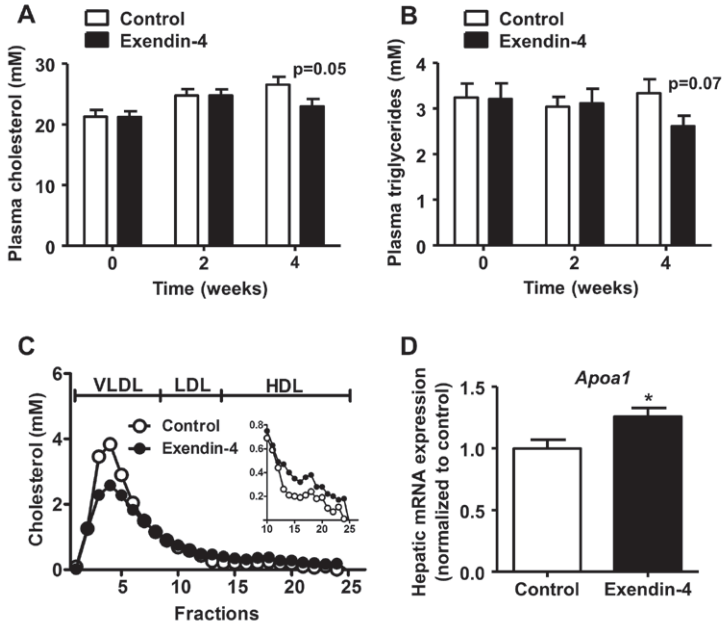


Figure 1. Exendin-4 decreases (V)LDL and slightly increases HDL. After 5 weeks of feeding Western-type diet containing 0.4% cholesterol, mice were treated with exendin-4 (50 µg/kg/day) or vehicle (PBS) subcutaneously for 4 weeks. Blood was collected by tail bleeding after 4 h of fasting before treatment (T=0) and after 2 (T=2) and 4 (T=4) weeks of treatment. Plasma cholesterol (A) and triglyceride (B) levels were determined. After 4 weeks of treatment, group-wise pooled plasma was fractionated using FPLC on a Superose 6 column and the individual fractions were assayed for cholesterol (C). Livers were isolated, mRNA was extracted and Apoa1 mRNA was determined as normalized to *Cyclo* and *Hprt* mRNA levels. Data were calculated as fold difference compared to vehicle (D). Values are means ± SEM (n=17 mice per group). **P*<0.05 compared to vehicle.

Exendin-4 decreases plasma VLDL and slightly increases HDL

Despite clear beneficial effects on glucose metabolism, exendin-4 only tended to decrease plasma cholesterol levels (-14%, *P*=0.05, Fig. 1A) and TG levels (-22%, *P*=0.07, Fig. 1B) after 4 weeks of treatment. Lipoprotein profiling revealed that exendin-4 decreased VLDL-cholesterol and slightly increased HDL-cholesterol levels (Fig. 1C). The latter effect was accompanied by a 26% increased hepatic expression of apolipoprotein A1 (*Apoa1*), encoding the major lipoprotein of HDL (Fig. 1D).

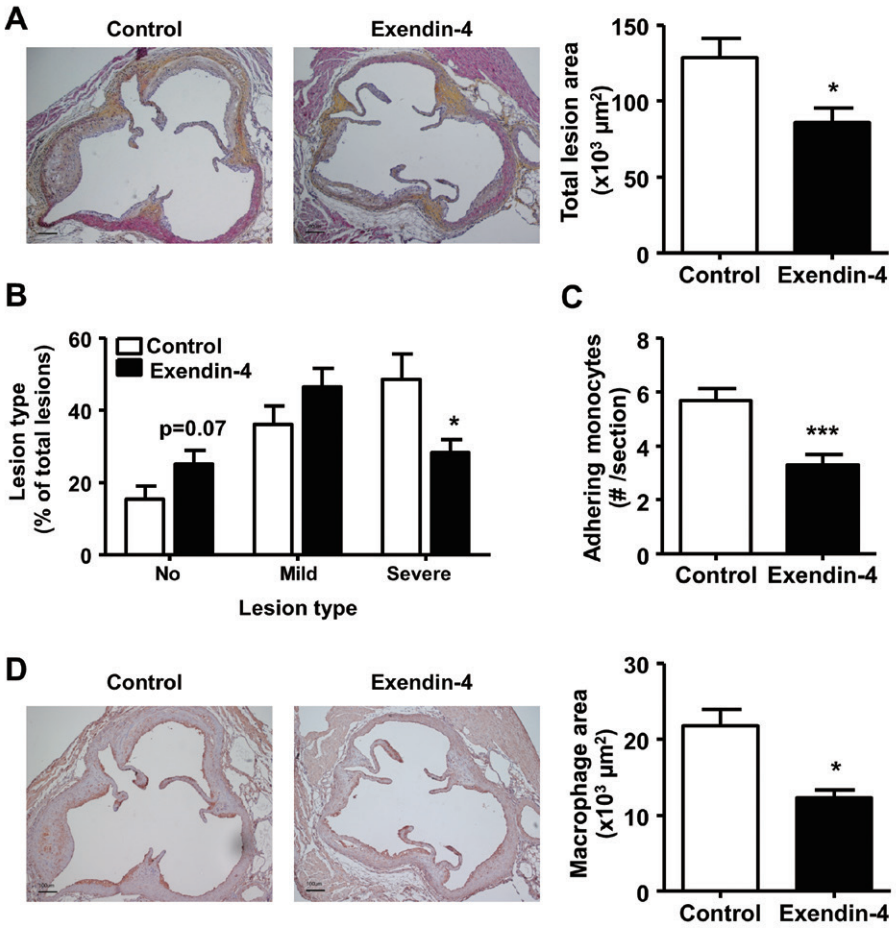


Figure 2. Exendin-4 reduces aortic atherosclerosis development and monocyte recruitment to the endothelium wall. After 5 weeks of feeding a Western-type diet containing 0.4% cholesterol, mice were treated with exendin-4 (50 µg/kg/day) or vehicle (PBS) subcutaneously for 4 weeks. Subsequently, hearts were isolated, fixed, dehydrated and embedded in paraffin. Cross-sections of the aortic root were stained with hematoxylin-phoxin-saffron (HPS) (A, B) or anti-AIA serum (C, D). Total lesion area (A), lesion severity (B), the number of adhering monocytes to the endothelium wall (C), and macrophage area (D) were quantified. Values are means ± SEM (n=17 mice per group). **P*<0.05, ****P*<0.001 compared to vehicle.

Exendin-4 largely suppresses atherosclerosis development and monocyte recruitment in the aortic root

Despite slightly attenuating dyslipidemia, exendin-4 treatment markedly reduced total atherosclerotic lesion area as compared to controls (-33%; $P<0.05$) (Fig. 2A). Additionally, mice treated with exendin-4 showed more lesion-free sections (+63%; $P=0.07$) and less severe lesions (-42%; $P<0.05$) as compared to PBS controls (Fig. 2B). Moreover, exendin-4 significantly reduced the number of monocytes adhering to the endothelium wall in the aortic root (-42%, $P<0.001$, Fig. 2C) as well as the macrophage area in the plaque (-44%, $P<0.05$, Fig. 2D).

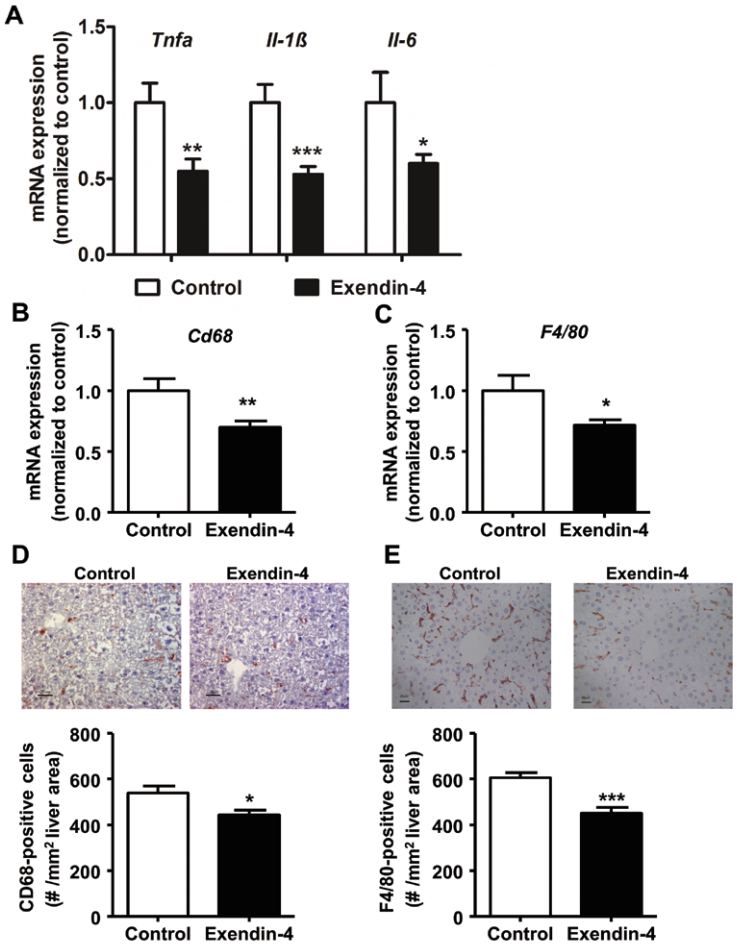


Figure 3. Exendin-4 reduces liver inflammation and macrophage content. After 5 weeks of feeding a Western-type diet containing 0.4% cholesterol, mice were treated with exendin-4 (50 µg/kg/day) or vehicle (PBS) subcutaneously for 4 weeks. mRNA was extracted from liver pieces, and mRNA expression of inflammatory markers *Tnfa*, *Il-1β*, and *Il-6* (A), *Cd68* (B) and *F4/80* (C) was determined as normalized to *Cyclo* and *Hprt* mRNA levels. Data were calculated as fold difference as compared to vehicle (A, B, C). Liver sections were immunostained for CD68 and F4/80, and $CD68^+$ (D) $F4/80^+$ (E) macrophages were quantified. Values are means ± SEM (n=17 mice per group). *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared to vehicle.

Exendin-4 reduces hepatic lipids, inflammation and macrophage content

Four weeks of exendin-4 treatment decreased hepatic TG (-11%; *P*=0.057) and TC content (-19%, *P*<0.05), without affecting hepatic phospholipid content. In addition, exendin-4 largely reduced the hepatic expression of the inflammatory markers *Tnfα*, *Il-1β*, and *Il-6* (-45%; *P*<0.01; -47%; *P*<0.001; -40%; *P*<0.05, respectively; Fig. 3A). Moreover, exendin-4 reduced hepatic mRNA expression of the macrophage markers *Cd68* (-30%; *P*<0.01; Fig. 3B) and *F4/80* (-28%; *P*<0.05; Fig. 3C). In line with these data, exendin-4 decreased hepatic CD68$^+$ macrophages (-18%, *P*<0.05; Fig. 3D) and F4/80$^+$ macrophages (-25%, *P*<0.001; Fig. 3E).

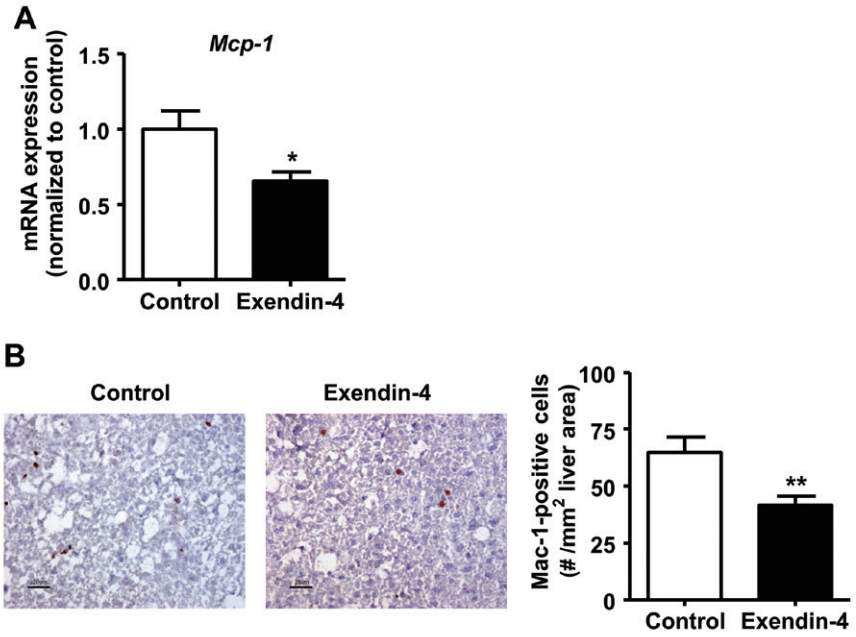


Figure 4. Exendin-4 reduces macrophage infiltration into the liver. After 5 weeks of feeding a Western-type diet containing 0.4% cholesterol, mice were treated with exendin-4 (50 µg/kg/day) or vehicle (PBS) subcutaneously for 4 weeks. In the livers, mRNA expression of Mcp-1 (A) were determined as normalized to *Cyclo* and *Hprt* mRNA levels. Data were calculated as fold difference as compared to vehicle (A). Liver sections were immunostained for Mac-1 and Mac-1$^+$ macrophages were quantified (B). Values are means ± SEM (n=17 mice per group). **P*<0.05, ***P*<0.01 compared to vehicle.

Exendin-4 reduces macrophage infiltration into the liver

To elucidate the mechanism underlying the reduction of liver macrophage content by exendin-4, we first determined the hepatic gene expression of monocyte chemotactic

protein-1 (*Mcp-1*), which mediates monocyte recruitment from the circulation to sites of infection and inflammation. As shown in Fig. 4A, exendin-4 reduced *Mcp-1* expression (-34%; *P*<0.05). This was accompanied by a reduction of macrophages positive for Mac-1, an infiltrating macrophage marker (-36%, *P*<0.01; Fig. 4B).

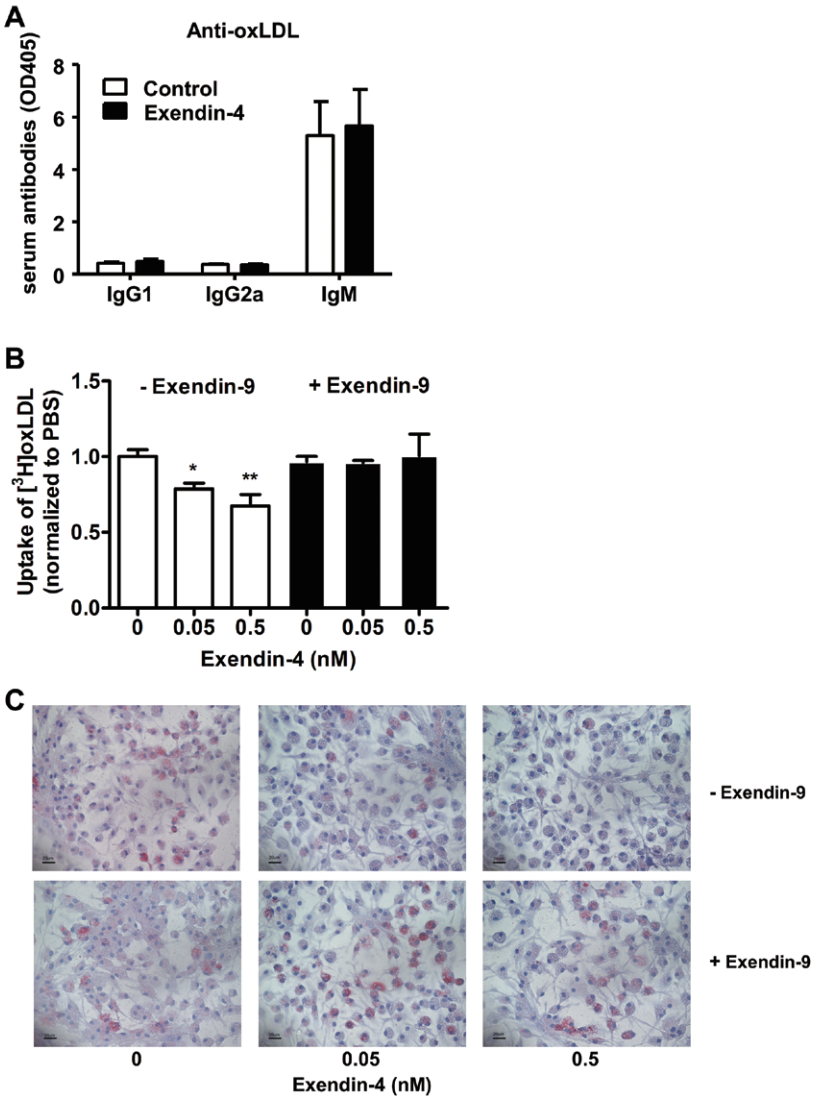


Figure 5. Exendin-4 has no effect on plasma antibodies against oxLDL, but reduces oxLDL-induced foam cell formation by using peritoneal macrophages. After 5 weeks of feeding a Western-type diet containing 0.4% cholesterol, mice were treated with exendin-4 (50 µg/kg/day) or vehicle (PBS) subcutaneously for 4 weeks. Plasma anti-oxidized LDL (oxLDL) antibodies were determined (A). Peritoneal macrophages were incubated with exendin-4 and/or exendin-9 for 48 h. The uptake of [^{3}H]COEth-labeled oxLDL was quantified (B). After fixation, lipid accumulation into the cells was visualized by staining with Oil red O (C). Values are means ± SEM (n=17 mice per group). **P*<0.05, ***P*<0.01 compared to vehicle.

Exendin-4 does not affect circulating antibodies against oxLDL, but reduces oxLDL uptake by macrophages

Since the uptake of oxLDL by macrophages can drive both atherosclerosis and NASH, we first measured specific antibodies against oxLDL in the circulation. Exendin-4 did not affect oxLDL-specific IgG1, IgG2a, and IgM levels in plasma (Fig. 5A). Next, to determine if exendin-4 can impact on oxLDL uptake by macrophages, we incubated peritoneal macrophages with [^{3}H]COEth-oxLDL (10 µg/ml) for 48 h with or without exendin-4 (0.05 and 0.5 nM). Exendin-4 decreased the uptake of [^{3}H]COEth-oxLDL (up to -33%, $P<0.01$) compared to controls (Fig. 5B). This effect was completely abolished by pre-treatment of the cells with exendin-9 (Fig. 5B), which demonstrates that exendin-4 reduces oxLDL uptake by activating the GLP-1R. Oil-red O staining confirmed that exendin-4 reduced foam cell formation, which was blocked by exendin-9 (Fig. 5C).

DISCUSSION

In this study, we investigated the effect of exendin-4 treatment on liver inflammation in addition to the development of atherosclerosis in *E3L.CETP* mice fed a Western-type diet. Our data show that 4 weeks of exendin-4 infusion markedly decreases total atherosclerotic lesion area, accompanied by a reduction in plaque macrophages. In parallel, exendin-4 caused a marked reduction in hepatic lipids and macrophages as well as hepatic inflammation, hallmarks of NASH.

It is interesting to note that short-term treatment with exendin-4, as compared with vehicle, caused a substantial reduction in atherosclerosis albeit that exendin-4 only slightly affected cholesterol levels and the lipoprotein profile. Therefore, the anti-atherogenic effect of exendin-4 is likely largely independent of modulation of plasma lipid levels. This observation contrasts the effects of classic lipid-lowering compounds including atorvastatin, which reduce atherosclerosis in *E3L.CETP* mice mainly by reducing apoB-containing lipoproteins [14]. We found that exendin-4 inhibited the adherence of monocytes to the vessel wall and decreased the macrophage area of the plaque, suggesting that exendin-4 decreases the recruitment of monocytes into the vessel wall. Our finding add to previous findings demonstrating that 4 weeks of GLP-1 receptor activation decreases atherosclerotic development without reducing plasma lipids in ApoE$^{-/-}$ mice [10, 18, 19]. Although a recent study did not find a reduction in atherosclerosis in ApoE$^{-/-}$ mice [11], this might be related to potential tissue heterogeneity in GLP-1 receptor expression in the ApoE$^{-/-}$ mice as well as variations in the experimental set-up (e.g. animal age, treatment regimen).

Second, we observed that exendin-4 reduced the lipid content of the liver, which

was accompanied by a reduction in inflammatory markers and macrophage content of the liver as judged from hepatic gene and protein expression. It is known that feeding hyperlipidemic mice a diet containing cholesterol increases the hepatic macrophage content already within a few days [20]. Since in our study mice were fed the Western-type diet for 5 weeks before starting treatment, exendin-4 may thus have reduced the hepatic macrophage content either by reducing the infiltration of activated macrophages from the circulation or by inducing the elimination of macrophages from the liver. The current study design does not allow us to discriminate between these possibilities. However, we did observe a decrease in hepatic MCP-1 expression along with a reduction in Mac-1^{+} infiltrating macrophages, suggesting that reduced recruitment of monocytes/macrophages from the circulation to the liver contributes to the reduction in total macrophages. Collectively, we show that exendin-4 treatment affects important features of NASH, including reduction of liver macrophages as well as lipid content.

Historically, NASH was thought to be a causal risk factor for cardiovascular disease (CVD) as patients with NASH have a higher mortality risk than the general population, mainly due to CVD [2]. However, the biological mechanisms linking NASH and accelerated atherosclerosis are still poorly understood. Recently, Bieghs *et al.* put forward the hypothesis that there is a central role for inflammation in the development of NASH and atherosclerosis with common etiologies involving monocyte recruitment and macrophage foam cell formation [3]. From this perspective, we hypothesized that exendin-4 reduces atherosclerosis as well as hepatic inflammation by acting directly on monocyte/macrophage recruitment into both the vessel wall and liver.

So what mechanisms may then be involved? In addition to reducing hepatic MCP-1 as observed in our study, exendin-4 reduces the expression of E-selectin, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 in the aorta of ApoE$^{-/-}$ mice [18]. These are all processes which likely lead to inhibition of monocyte/macrophage adhesion to the vessel wall and the liver. The importance of blood monocytes in the development of atherosclerosis has been firmly established [21], and accumulating evidence demonstrates the role of hepatic infiltration of blood-borne monocytes in liver inflammation [22, 23]. In fact, the migration of immune cells into the liver appears to be one of the first critical steps in both acute and chronic liver inflammation, mediating innate and adaptive inflammatory responses, which result in ongoing immune cell recruitment, tissue damage, and fibrosis [23].

As a second mechanism explaining the reduction of macrophage recruitment, we showed that exendin-4, via the GLP-1R, reduces the uptake of oxLDL by peritoneal macrophages and, as a consequence, reduced foam cell formation in vitro. These data are in full accordance with previous observations showing that native GLP-1 also

reduces oxLDL uptake by peritoneal macrophages thereby reducing atherosclerosis development from ApoE$^{-/-}$ mice [19]. We did not observe any changes in plasma anti-oxLDL antibodies after exendin-4 treatment, indicating that exendin-4 probably does not affect the oxLDL levels in plasma. Since native GLP-1 decreased the macrophage protein levels of CD36 [19], involved in the uptake of modified LDL, it is likely that in our study exendin-4 exerts its protective effects on macrophage foam cell formation by a similar mechanism. The relevance for oxLDL uptake via scavenger receptors by macrophages in atherosclerosis development has been confirmed by many studies [24]. Recent studies have shown that oxLDL uptake via scavenger receptors is also an important risk factor for the progression to hepatic inflammation [15]. In fact, inhibition of oxLDL uptake and thus foam cell formation by deficiency of the scavenger receptors CD36 and scavenger receptor class A in hematopoietic cells reduces hepatic macrophage infiltration [25], confirming that oxLDL uptake by macrophages not only plays a vital role in atherosclerosis development but also regulates hepatic inflammation.

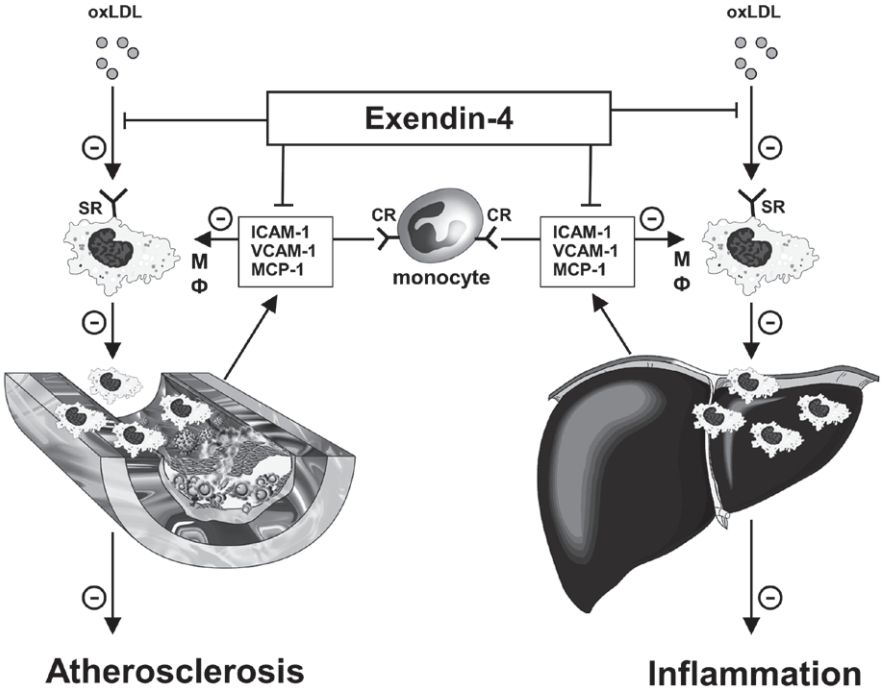


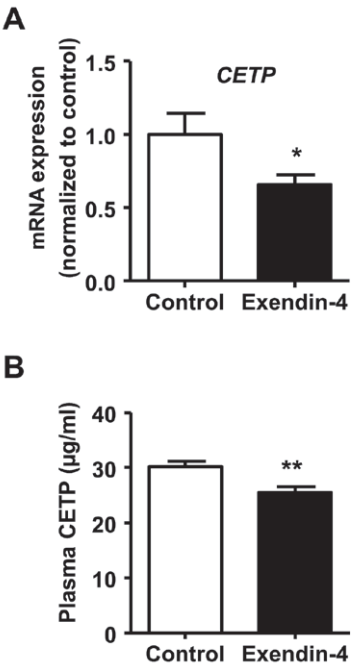
Figure 6. Proposed mechanism underlying the beneficial effects of exendin-4 on atherosclerosis development and NASH. Exendin-4 attenuates the development of atherosclerosis and NASH by 1) reducing the expression of chemokines and adhesion molecules, which leads to less recruitment of circulating monocytes/macrophages, and 2) inhibiting the uptake of oxLDL by macrophages and the subsequent formation of foam cells in the vessel wall and liver, respectively. For further explanation see text. CR, chemokine receptor(s); ICAM, intercellular adhesion molecule-1; mΦ, macrophage; MCP-1, monocyte chemotactic protein-1; oxLDL, oxidized LDL; SR, scavenger receptor; VCAM, vascular cell adhesion molecule-1.

Collectively, we hypothesize that the protective effect of exendin-4 against inflammation in both the vessel wall and liver can be explained by (1) reducing monocyte/macrophage recruitment from the circulation and by (2) inhibiting the uptake of oxLDL by macrophages and the subsequent activation and formation of foam cells at these locations (see Fig. 6).

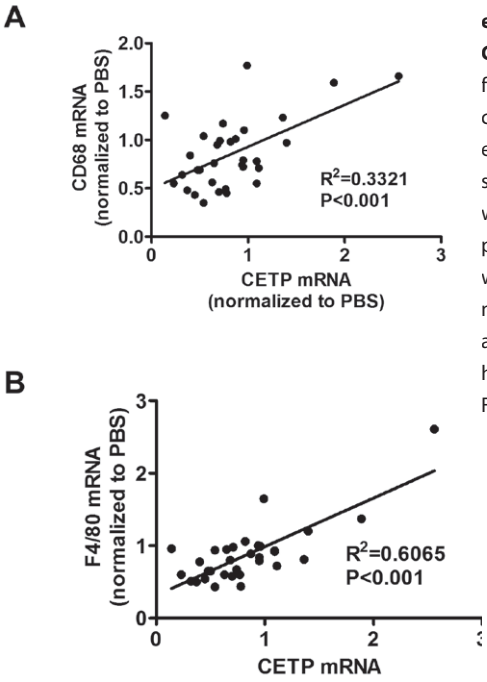
Interestingly, we also observed that exendin-4 decreased hepatic CETP gene expression (-34%, $P<0.05$) and plasma CETP concentration (-15%, $P<0.01$) (Supplemental Fig. S2). Recently, we demonstrated similar effects for niacin, which were explained by a reduction in macrophages that largely contribute to hepatic CETP expression [26]. In our present study, hepatic CETP expression positively correlated with the hepatic expression of the macrophage markers CD68 ($R^2=0.332$; $P<0.001$) and F4/80 ($R^2=0.607$; $P<0.001$) (Supplemental Fig. S3), indicating that exendin-4 also reduces CETP expression by reducing the hepatic macrophage content. Since CETP is involved in the transfer of cholesteryl esters from HDL to (V)LDL, the reduction in CETP may have contributed to the slight decrease in (V)LDL/HDL ratio, and thereby to reduced atherosclerosis development. Taken together, these data suggest that reducing NASH in general, by reducing the macrophage content, may reduce CETP expression, thereby improving the lipoprotein profile and therefore decrease the risk of developing atherosclerosis.

Thus far, there is no established pharmacological compound to treat NASH. Lifestyle modifications, such as weight loss, exercise, and restriction of nutrition intake are still the mainstays for the treatment of NASH [27]. Although lipid-lowering agents (e.g. statins and fibrates) and anti-oxidants (e.g. vitamins C, E) have beneficial effects on atherosclerosis development, none of them have shown adequate and convincing benefits in the treatment of NASH [28, 29]. Exendin-4 has been approved for the treatment of T2DM [5] and has also been shown to possess cardioprotective actions that include, in addition to reducing atherogenesis, suppression of arrhythmias, heart failure, myocardial infarction, and death [30]. Based on our collective findings that exendin-4 reduces high fat diet-induced hepatic steatosis by decreasing lipogenesis [6] and suppresses macrophage content in both vessel wall and liver (in the current study), we propose that exendin-4 is a suitable candidate to concomitantly treat atherosclerosis and NASH.

In conclusion, our findings show that exendin-4 treatment reduces inflammation in both the liver and vessel wall in *E3L.CETP* mice fed a Western-type diet, by reducing monocyte/macrophage recruitment and inhibiting the uptake of oxLDL by macrophages. We anticipate that exendin-4 can be used as a valuable strategy to treat NASH and atherosclerosis in addition to T2DM, especially in patients who display a combination of these diseases.



Supplemental Figure S2. Exendin-4 decreases hepatic expression and plasma level of CETP. After 5 weeks of feeding a Western-type diet containing 0.4% cholesterol, mice were treated with exendin-4 (50 µg/kg/day) or vehicle (PBS) subcutaneously for 4 weeks. mRNA was extracted from liver pieces, and mRNA expression of *CETP* (A) was determined as normalized to *Cyclo* and *Hprt* mRNA levels. Data were calculated as fold difference as compared to vehicle. Plasma CETP levels were determined (B). Values are means ± SEM (n=17 mice per group). **P*<0.05, ***P*<0.01 compared to vehicle.



Supplemental Figure S3. Hepatic CETP expression is positively correlated with hepatic CD68 and F4/80 expression. After 5 weeks of feeding a Western-type diet containing 0.4% cholesterol, *E3L.CETP* mice were treated with exendin-4 (50 µg/kg/day) or vehicle (Control) subcutaneously for 4 weeks. Subsequently, livers were isolated and mRNA was extracted from liver pieces. mRNA expression of *Cd68*, *F4/80*, and *CETP* was determined as normalized to *Cyclo* and *Hprt* mRNA levels. Data were calculated as fold difference as compared to vehicle and the correlation between hepatic CETP expression and hepatic CD68 (A) or F4/80 (B) expression was linearly plotted.

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