Activation of CXCR7 Limits Atherosclerosis and Improves Hyperlipidemia
by Increasing Cholesterol Uptake in Adipose Tissue

Running title: Li et al.; CXCR7 in atherosclerosis and hyperlipidemia

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Abstract

Background—The aim of this study was to determine the role of the chemokine receptor CXCR7 in atherosclerosis and vascular remodeling. CXCR7 is the alternative receptor of CXCL12, which regulates stem cell-mediated vascular repair and limits atherosclerosis via its receptor CXCR4.

Methods and Results—Wire-induced injury of the carotid artery was performed in mice with a ubiquitous, conditional deletion of CXCR7 and in mice treated with the synthetic CXCR7 ligand CCX771. The effect of CCX771 treatment on atherosclerosis was studied in Apoe−/− mice fed a high fat diet for 12 weeks. Lipoprotein fractions were quantified in the plasma of Apoe−/− mice by FPLC. Uptake of Dil-labeled VLDL to adipose tissue was determined by 2-photon microscopy. We show that genetic deficiency of Cxcr7 increased neointima formation and lesional macrophage accumulation in hyperlipidemic mice after vascular injury. This was related to increased serum cholesterol levels and subsequent hyperlipidemia-induced monocytosis. Conversely, administration of the CXCR7 ligand, CCX771, to Apoe−/− mice inhibited lesion formation and ameliorated hyperlipidemia following vascular injury and during atherosclerosis. Treatment with CCX771 reduced circulating VLDL levels, but not LDL or HDL levels, and increased uptake of VLDL into Cxcr7-expressing white adipose tissue. This effect of CCX771 was associated with an enhanced lipase activity and reduced Angptl4 expression in adipose tissue.

Conclusions—CXCR7 regulates blood cholesterol by promoting its uptake in adipose tissue. This unexpected cholesterol-lowering effect of CXCR7 is beneficial for atherosclerotic vascular diseases presumably by ameliorating hyperlipidemia-induced monocytosis and can be augmented using a synthetic CXCR7 ligand.

Key words: atherosclerosis, hypercapnia, hypercholesterolemia, restenosis
Chemokines, acting through various mechanisms including the recruitment of immune cells and smooth muscle progenitor cells (SPCs) to the vessel wall\(^1,2\), are crucial for vascular remodeling and atherosclerosis. In addition, chemokines are critical for monocyte and neutrophil homeostasis. Hyperlipidemia-induced monocytosis results from the combined action of various chemokine receptors, such as CCR2, which are expressed by immune cells (including monocytes), and facilitates atherosclerosis\(^3-5\). By contrast, the CXCL12 receptor, CXCR4, protects against atherosclerosis by controlling the mobilization of neutrophils\(^6\), whereas the mobilization and recruitment of SPCs by CXCL12 during vascular repair aggravates neointima formation, indicating a context-specific role for this chemokine-receptor axis in arterial remodeling\(^7,8\). Accordingly, increased levels of CXCL12 and SPCs are observed in patients with severe cardiac allograft vasculopathy\(^9\). Systemic treatment with CXCL12 induces the release of SPCs, which accumulate in atherosclerotic lesions and thus lead to plaque stabilization\(^10\).

Whereas most of the effects of CXCL12 are linked to its interaction with CXCR4, the function of the alternative CXCL12 receptor, CXCR7, in atherogenesis and vascular remodeling is unclear.

CXCR7 binds to both CXCL12 and CXCL11 and promotes the growth and adhesion of tumor cells\(^11,12\). Genetic deletion of Cxcr7 in mice results in a high level of perinatal lethality and abnormal cardiovascular development\(^13,14\). In contrast to other chemokine receptors, CXCR7 is not expressed on leukocytes\(^15\). Although structurally characterized as a G protein-coupled receptor (GPCR), CXCR7 does not induce classical GPCR signaling events, but can trigger the recruitment of \(\beta\)-arrestin-2, which leads to receptor internalization or activation of downstream signaling pathways, e.g., MAP kinases\(^16,17\). The interaction between CXCR7 and \(\beta\)-arrestin-2 controls the bioavailability of extracellular CXCL12\(^18,19\). Furthermore, cross-regulation of
CXCR4 by CXCR7 can affect CXCR4-dependent Gαi protein activation. Interestingly, the highly selective synthetic CXCR7 ligand CCX771 antagonizes the binding of CXCL12 to CXCR7 but induces the association of β-arrestin-2 with CXCR7 more effectively than its endogenous ligands. In light of this complex interplay between CXCL12, CXCR4, and CXCR7, we aimed to dissect the role played by CXCR7 during vascular remodeling and atherosclerosis and to assess the potential interactions between CXCR7 and the CXCL12/CXCR4 axis in vascular disease.

Here, we identify a key role for CXCR7 in the regulation of serum cholesterol levels by enhancing the uptake of VLDL and increasing the uptake of cholesterol in adipose tissue. The cholesterol-lowering effect of CXCR7 was associated with a reduction in hyperlipidemia-induced monocytosis, and with decreased arterial lesion formation and macrophage accumulation.

Methods

Mice

Cxcr7LacZ/+ and Cxcr7flox mice (generated on a pure C57Bl/6 background) were provided by ChemoCentryx, Inc., Mountain View, CA, USA. CAG-CreERTM, Apoe-/-, and Ldlr-/- mice were obtained from The Jackson Laboratory (Bar Harbor, Maine, USA). Mice with a ubiquitous tamoxifen (TMX)-inducible Cxcr7 knockout were generated by breeding CAG-CreERTM×Cxcr7WT/WT with Cxcr7flox/flox mice. CAG-CreERTM×Cxcr7flox/flox mice were further crossed with Apoe-/- mice to obtain CAG-CreERTM×Cxcr7flox/flox Apoe-/- mice.

Animal models

Wire injury of carotid arteries was performed in CAG-CreERTM×Cxcr7flox/flox and CAG-
CreERT<sup>TM</sup>Cxcr<sup>7flox/flox</sup> Apoe<sup>−/−</sup> and Apoe<sup>−/−</sup> mice fed a high-cholesterol diet (HCD; 21% fat and 0.15% cholesterol; Altromin, Lage, Germany), as previously described<sup>7</sup>. Diet-induced atherosclerosis was induced by feeding Apoe<sup>−/−</sup> mice a HCD for 12 weeks. Treatment with CCX771 was given via a daily subcutaneous injection (10 mg/kg/d dissolved in 10% Captisol; 100 μl per injection). Control mice were injected with Captisol alone (10%; 100 μl/mouse, s.c.). Mice expressing TMX-inducible Cre recombinase and the floxed Cxcr7 allele, and the corresponding control mice, were treated with TMX (1.5 mg/20 g BW; Sigma-Aldrich Chemie GmbH, Munich, Germany) dissolved in neutral oil (MIGYOL, Sasol Germany GmbH, Hamburg, Germany) for 5 consecutive days (i.p. injection). Wire injury of the carotid arteries was performed 15 days after the last injection of TMX. One week prior to vascular injury, splenectomy was performed in some Apoe<sup>−/−</sup> mice after ligation of the splenic vessels. Bone marrow cells were harvested from femurs of CAG-Cre<sup>+</sup>Cxcr<sup>7flox</sup> Apoe<sup>−/−</sup> or CAG-Cre Cxcr<sup>7flox</sup> Apoe<sup>−/−</sup> mice and injected (4 x 10<sup>6</sup> cells) into the tail vein of Apoe<sup>−/−</sup> recipient mice 24 h after total body irradiation (2 × 6 Gy, 4 h interval). Lipid metabolism was studied in Apoe<sup>−/−</sup> and Ldlr<sup>−/−</sup> mice fed a HCD after 4 weeks of treatment with CCX771 or Captisol.

The aorta and carotid arteries were harvested following in situ perfusion fixation with 4% paraformaldehyde (PFA, Carl Roth GmbH, Karlsruhe, Germany) or PAXgene (Qiagen GmbH, Hilden, Germany). All animal experiments were reviewed and approved by the local authorities (NRW LANUV) according to German animal protection laws.

**Histomorphometry**

Serial (4 μm-thick) cross-sections of the left common carotid arteries [within a standardized distance (80 to 320 μm) from the bifurcation] and the aortic roots were obtained and subsequently stained with modified Movat’s pentachrome or elastic van Gieson (EvG) stain (4-6
sections per mouse). Lesions and medial areas were determined by planimetry of digitized images using Diskus software (Hilgers, Königswinter, Germany). The thoracoabdominal aortas were en face prepared and stained with oil red O. The oil red O-stained area and the total aortic surface area were quantified using ImageJ software.

**Immunostaining**

The expression of CXCR7 was studied in 4% PFA-fixed cryosections following antigen retrieval with citrate buffer (20 min heating in a microwave oven) by incubating with an antibody against CXCR7 (clone 11G8, a gift from ChemoCentryx, Inc.). A secondary FITC-conjugated anti-mouse antibody (polyclonal goat, Jackson ImmunoResearch Europe Ltd., Suffolk, UK) was used to visualize the primary antibody. Sections from Cxcr7−/− mice were used for negative control staining.

The composition of the lesions was determined by immunostaining of α-smooth muscle actin (α-SMA; clone 1A4; Dako Deutschland GmbH, Hamburg, Germany) and macrophage-specific Mac2 (clone M3/38; Cedarlane, Burlington, Canada) in paraffin-embedded carotid arteries and aortic roots. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Vectashield, Vector Laboratories). Non-specific antibodies (Santa Cruz Biotechnology) were used for negative control staining. A secondary DyLight 594-conjugated antibody (polyclonal goat; Jackson ImmunoResearch Europe Ltd.) was used to visualize the primary antibody. Digital images from at least four sections per mouse were recorded using a fluorescence microscope (Leica DM 2500; Leica Microsystems GmbH, Wetzlar, Germany) equipped with a CCD camera (JVC Deutschland GmbH, Friedberg, Germany) using Diskus software. The number Mac2-stained macrophages and α-SMA-stained SMCs within the lesions were determined by counting the number of nuclei surrounded by positive staining for Mac2 and α-SMA, respectively.
Statistical analysis

Data represent the mean ± S.E.M. Paired or unpaired Student's t-test, one-way ANOVA (followed by the Newman-Keuls post-test), or two-way ANOVA (followed by uncorrected Fisher’s Least Significant Difference) were used for statistical comparison between groups (Prism 5, GraphPad Software, La Jolla, USA). P values < 0.05 were considered statistically significant.

Results

Cxcr7 expression patterns

Transcripts for both Cxcr7 and Cxcr4 were highly expressed in lung, spleen, and adipose tissue compared with liver and skeletal muscle (Supplemental Figure 1A and B). In contrast to Cxcr4, the expression of Cxcr7 was also higher in arterial tissue, heart, and kidney compared with blood and bone marrow (BM) cells (Supplemental Figure 1A and B). CXCR7 was detected on cultured endothelial cells by flow cytometry, but not on murine peripheral leukocytes, BM cells, or platelets (Supplemental Figure 1C). X-gal staining of tissues from adult Cxcr7+/+LucZ mice showed expression of Cxcr7 in the marginal and/or sinusoidal zones of the spleen, in epithelial or endothelial cells of the kidney and lung, in cells of the intestinal lamina propria, in adipocytes, and in cardiomyocytes, but not in hepatocytes (Supplemental Figure 2). Moreover, activation of the Cxcr7 promoter was observed in endothelial and adventitial cells of the aorta, and in the endothelium covering the aortic valves (Supplemental Figure 2). Expression of CXCR7 protein was detected in the spleen, kidney, and endothelium of the aortic valves by immunostaining (Supplemental Figure 3), but not in the carotid artery wall (Supplemental Figure 4).

Conditional Cxcr7 deletion exacerbates neointimal hyperplasia

Ubiquitous deletion of Cxcr7 was induced in Apoe−/− (CAG-Cre+Cxcr7floxApoe−/−) mice by
tamoxifen (TMX) treatment. Knockdown of Cxcr7 in CAG-Cre+ Cxcr7flox mice was verified by CXCR7 immunostaining of the spleen (Figure 1A). Next, wire injury of the carotid artery was performed in CAG-Cre+ Cxcr7flox Apoe−/− mice fed a HCD. The neointimal, but not the medial, area was increased at 28 d after vascular injury in CAG-Cre+ Cxcr7flox Apoe−/− mice compared with that in TMX-treated CAG-Cre− Cxcr7flox Apoe−/− mice (Figure 1B). The lengths of the internal (IEL) and external elastic laminae (EEL) were similar in both groups (Figure 1B). The number of Mac2+ macrophages was higher in CAG-Cre+ Cxcr7flox Apoe−/− mice than in CAG-Cre− Cxcr7flox Apoe−/− mice, as determined by Mac2 immunostaining (Figure 1C). Although the number of smooth muscle cells (SMCs) in the neointima tended to increase in CAG-Cre+ Cxcr7flox Apoe−/− mice, the difference was not statistically significant (Figure 1D). Moreover, the α-SMA+ neointimal area was comparable between CAG-Cre+ Cxcr7flox Apoe−/− and CAG-Cre− Cxcr7flox Apoe−/− mice (Figure 1D).

Mobilization of SPCs after vascular injury is mediated by CXCL121. However, the injury-induced increase of circulating Sca-1+Lin− cells was unaffected in CAG-Cre− Cxcr7flox Apoe−/− mice compared with that in CAG-Cre+ Cxcr7flox Apoe−/− mice (Figure 2A). Moreover, CXCL12 levels in the plasma and BM of CAG-Cre+ Cxcr7flox Apoe−/− mice were higher than those in CAG-Cre+ Cxcr7flox Apoe−/− mice at 28 d after injury (Figure 2B). The expression of Cxcr4 mRNA in various tissues, such as blood cells, aorta, and adipose tissue, was not altered in CAG-Cre+ Cxcr7flox Apoe−/− mice (Figure 2C). Notably, neointima formation did not differ between CAG-Cre+ Cxcr7flox Apoe−/+ mice and CAG-Cre+ Cxcr7flox Apoe−/+ mice fed a HCD diet (Figure 2D), indicating that the effect of CXCR7 is related to serum cholesterol levels. Apoe−/− mice showed markedly higher serum cholesterol levels than Apoe−/+ mice (Figure 2E). Whereas serum cholesterol and triglyceride (TG) concentrations were further increased in CAG-
Cre+Cxcr7floxApoe−/− mice at 28 d after injury compared with those in CAG-Cre Cxcr7floxApoe−/− mice, cholesterol and TG levels in CAG-Cre+Cxcr7floxApoe+/+ mice and CAG-Cre+Cxcr7floxApoe−/− mice did not differ (Figure 2E). Similarly, circulating monocyte counts were higher in Apoe−/− mice than in Apoe+/+ mice, and were further increased in CAG-Cre+Cxcr7floxApoe−/− mice (but not in CAG-Cre+Cxcr7floxApoe+/+ mice) when compared with the respective control groups (Figure 2F). In contrast, the neointimal area, serum cholesterol and TG levels, and peripheral monocyte counts in TMX-treated Apoe−/− mice harboring CAG-Cre+Cxcr7floxApoe−/− BM cells were not substantially different from the control group (Supplemental Figure 5). These findings suggest that the effect of Cxcr7 deficiency on neointima formation was due to increased serum cholesterol levels, which indirectly leads to monocytosis.

To study the effects of deletion of the Cxcr7 gene on body composition, adipose tissue (AT) size was analyzed in CAG-Cre+Cxcr7floxApoe−/− and CAG-Cre+Cxcr7floxApoe+/+ mice fed a HCD for 4 wk by micro-CT. The total and lean body weight and the body fat mass were similar between CAG-Cre+Cxcr7floxApoe−/− and CAG-Cre+Cxcr7floxApoe−/− mice (Figure 2G). Moreover, the visceral adipose (VAT) and the brown adipose tissue (BAT) mass were not significantly different between CAG-Cre+Cxcr7floxApoe−/− and CAG-Cre+Cxcr7floxApoe−/− mice (Supplementary Figure 6). In addition, the fasting glucose and insulin levels, and the surrogate marker for insulin sensitivity, QUICKI, were similar between both groups (Figure 2H).

CCX771 ameliorates neointimal lesion formation

To study the effect of a CXCR7 ligand in lesion formation, CCX771 (10 mg/kg) was injected to Apoe−/− mice reaching mean CCX771 plasma levels of 336 ng/ml after 1 h and 106 ng/ml after 12 h (Supplemental Figure 7A). Contrary to the effects of Cxcr7 deficiency, treatment with CCX771
reduced the neointimal area (Figure 3A and B) and macrophage accumulation as assessed by the number of lesional Mac2+ macrophages (Figure 3D) after wire injury in Apoe<sup>−/−</sup> mice fed a HCD compared with vehicle-injected controls. The medial area (Figure 3B), the lengths of the IEL and EEL (Figure 3C), and the neointimal SMC content as determined by the number of SMA<sup>+</sup> cells or the SMA<sup>+</sup> area (Figure 3E) did not differ between CCX771- and vehicle-treated mice. Treatment with CCX771 did not alter the injury-induced mobilization of Sca-1<sup>+</sup>Lin<sup>−</sup> SPCs (Figure 4A) or CD34<sup>+</sup>c-kit<sup>+</sup>Lin<sup>−</sup> EPCs (Figure 4B). Similar to Cxcr7<sup>−/−</sup> mice, the CXCL12 levels in the plasma and BM were also higher in CCX771-treated mice than in vehicle-treated mice (Figure 4C). However, treatment with CCX771 for 4 wk did not increase CXCL12 levels in the plasma and BM of Cre<sup>+</sup>Cxcr7<sup>fl<sub>ox</sub></sup>Apoe<sup>−/−</sup> mice compared with vehicle-injected controls (Supplemental Figure 8). Furthermore, serum cholesterol and TG levels (Figure 4D) and peripheral monocyte counts (Figure 4E) were reduced in CCX771-treated mice compared with those in vehicle-treated mice. The Gr-1<sup>high</sup>/Gr-1<sup>low</sup> monocyte ratio was unaffected by CCX771 treatment (Figure 4F). Aspartate transaminase (AST), and creatinine levels were not different between the groups (Supplemental Figure 7B and C).

To study whether Cxcr7 expression in the spleen plays a role in the effect of CCX771 on neointima formation and hypercholesterolemia, splenectomized Apoe<sup>−/−</sup> mice were treated with CCX771 after vascular injury. However, even in the absence of the spleen, treatment with CCX771 reduced both the neointimal area (Figure 4G) and serum cholesterol levels (Figure 4F).

**CCX771 lowers cholesterol levels and promotes VLDL uptake in adipose tissue**

To determine the role of CXCR7 in lipoprotein metabolism, lipoprotein profiles were analyzed in uninjured Apoe<sup>−/−</sup> mice fed a HCD after treatment with CCX771 or vehicle for 4 wk. Treatment
with CCX771 reduced the serum cholesterol and TG concentrations in fasting (5 h) and non-fasting mice, but did not affect the postprandial increase of cholesterol and TG levels (Figure 5A and B). The decrease in total serum cholesterol levels observed in CCX771-treated mice was due to a reduced concentration of circulating VLDL-cholesterol; however, LDL-cholesterol and HDL-cholesterol concentrations were unaltered by CCX771 (Figure 5C). In Ldlr⁻/⁻ mice, treatment with CCX771 also reduced the serum cholesterol and TG levels after 4 wk of a HCD compared with vehicle-treated controls (Supplemental Figure 9).

To study the VLDL-lowering mechanism of CCX771, hepatic TG secretion was analyzed by measuring the increase of serum TG levels after injection of the lipase inhibitor P-407²². At 6 h after P-407 administration, the TG levels increased to similar levels in CCX771- and vehicle treated mice (Supplemental Figure 10). Accordingly, the hepatic TG production rate was not altered by treatment with CCX771 (Figure 5D). To investigate the effect of CCX771 on the VLDL clearance, DiI-labeled human VLDL was injected to Apoe⁻/⁻ mice treated with CCX771 or vehicle for 4 wk. At 60 min after the injection of DiI-VLDL, the human ApoB100 and the DiI levels in the plasma were significantly reduced in CCX771-treated mice (Figure 5E), indicating that CCX771 enhances the clearance of VLDL.

Next, the DiI-VLDL uptake was determined in VAT, BAT, skeletal muscle, and the liver of Apoe⁻/⁻ mice. The concentration of DiI was significantly increased in VAT, but not in the liver, the BAT, and skeletal muscle of CCX771-treated compared with vehicle-treated mice (Figure 6A, B and Supplemental Figure 11). Accordingly, the DiI fluorescent signal in adipocytes was enhanced in CCX771-treated mice, as assessed by in vivo multi-photon microscopy (Figure 6C). Moreover, the cholesterol content was increased in VAT, but not in the liver, of CCX771-treated mice (Figure 6A and B). Uptake of DiI-VLDL to the subendothelial space was not detectable in
the carotid arteries of CCX771-treated mice and Cre$^+\text{C}xcr7^{\text{flo}}\text{x} Apoe^{−/−}$ mice by multi-photon and epifluorescence microscopy, respectively (Supplemental Figure 12). Taken together, these findings indicate that CXCR7 promotes the uptake of VLDL-derived cholesterol to adipose tissue.

Effects of CCX771 on adipose tissue and insulin sensitivity

To study the mechanism of CXCR7 mediated uptake of VLDL, human SGBS adipocytes, which expressed CXCR7, as detected by immunostaining (Supplemental Figure 13), were treated with Dil-VLDL. CCX771 significantly increased the basal uptake of Dil-VLDL as compared to untreated, vehicle-, or CCX704 (an analog of CCX771 with low affinity for CXCR7)-treated adipocytes (Figure 6D). Silencing of β-arrestin-2 using siRNAs (Figure 6E) prevented the uptake of Dil-VLDL triggered by CCX771 (Figure 6F), indicating that β-arrestin-2 promotes VLDL uptake to adipocytes mediated by CXCR7.

To assess the effect of CCX771 on lipoprotein lipase (LPL), which plays an important role in the clearance of VLDL\textsuperscript{23}, lipase activity was determined in Apoe\textsuperscript{−/−} mice after treatment with CCX771 or vehicle. The lipase activity was increased in VAT, but not in the plasma of CCX771- compared with vehicle-treated mice (Figure 7A). Angptl3 and Angptl4 are negative regulators of lipoprotein lipase activity\textsuperscript{24}. Treatment with CCX771 reduced Angptl4 protein levels in VAT but not in the plasma (Figure 7B), whereas Angptl3 protein levels were unaltered in the VAT and in the plasma in CCX771-treated as compared to vehicle-treated mice (Figure 7C). Moreover, the Angptl4 mRNA expression level was diminished in the VAT of CCX771-treated mice, while the expression of Lpl and Angptl3 transcripts remained unchanged (Figure 7D), indicating that down-regulation of Angptl4 may increase lipase activity in the VAT of CCX771-treated mice. In addition, the mRNA expression level of Pparg, a positive regulator of
Angptl4 expression in adipocytes, was reduced in the VAT of CCX771-treated mice (Figure 7D). However, treatment with CCX771 did not substantially change the expression of Vldlr and Ldlr transcripts in the VAT (Supplemental Figure 14A). Unlike Cxcr4, the expression of Cxcr7 in different VATs was comparable with that in the spleen (Supplemental Figure 14B).

To determine the impact of CCX771 on insulin sensitivity, Apoe<sup>−/−</sup> mice were fed a HCD and treated with CCX771 or vehicle for 4 wk. There was no significant difference in fasting and non-fasting blood glucose levels detectable between CCX771-treated and vehicle-treated mice (Figure 7E). The fasting insulin levels in the plasma were also similar between both groups (Figure 7F). Accordingly, the quantitative insulin-sensitivity check index (QUICKI) was not different between CCX771- and vehicle-treated mice (Figure 7G), indicating that the effects of CCX771 on adipose tissue do not affect insulin sensitivity.

**Treatment with CCX771 inhibits atherosclerosis**

To investigate the role of CCX771 in diet-induced atherosclerosis, Apoe<sup>−/−</sup> mice fed a HCD were treated with CCX771 or vehicle for 12 wk. The size of the atherosclerotic lesions in the aortic root (Figure 8A) and the thoracoabdominal aorta (Figure 8B), as well as the number of Mac2<sup>+</sup> macrophages in the aortic root lesions (Figure 8C), was reduced by CCX771. However, the number of SM22<sup>+</sup> SMCs and the SM22<sup>+</sup> area in the lesions were unaltered by CCX771 (Figure 8D), and serum cholesterol and TG levels (Figure 8E), as well as the peripheral monocyte count (Figure 8F) were reduced. Alanine transaminase (ALT), and creatinine levels (Supplemental Figure 15) were comparable between CCX771- and vehicle-treated mice.

**Discussion**

Our results indicate a key role for CXCR7 in regulating lipoprotein metabolism and serum
cholesterol levels in mice by enhancing the clearance of VLDL through adipose tissue under conditions of hyperlipidemia, thereby unveiling a new functional feature of chemokine receptors. Treatment with the CXCR7 ligand CCX771 reduced hyperlipidemia and subsequent monocytosis in Apoe<sup>−/−</sup> mice, thereby limiting atherosclerotic lesion formation. Thus, treatment with agents that activate CXCR7 appears to be a promising adjunct to lipid-lowering drugs that are used to prevent atherosclerosis, especially in the context of metabolic disorders characterized by increased VLDL levels.

The neointimal accumulation of SMCs after vascular injury is mediated by the CXCL12-dependent recruitment of SPCs from the BM<sup>1,8</sup>. CXCR4 is expressed on SPCs, and inhibition of CXCR4 or CXCL12 prevents neointima formation<sup>7, 8, 25</sup>. Our own results did not indicate that CXCR7 regulates SPC mobilization or neointimal SMC accumulation. Although CXCR7 is not expressed on circulating leukocytes<sup>15</sup>, we found that a genetic deficiency of Cxcr7 results in enhanced neointima formation due to increased accumulation of Mac2<sup>+</sup> macrophages. Alternatively, Mac2 (also known as galectin-3) may accumulate in the extracellular space after being released from macrophages<sup>36</sup>. However, changes of the lesional Mac2<sup>+</sup> macrophages content correlate very well with those of F4/80<sup>+</sup> macrophages, as determined by flow cytometry<sup>10</sup>. The increased macrophage accumulation can be explained by findings showing that the monocytosis induced by severe hyperlipidemia<sup>3, 5</sup> is aggravated in conditional Cxcr7<sup>−/−</sup> Apoe<sup>−/−</sup> mice. In addition, hyperlipidemia may also promote the transformation of SMCs into foam cells and the expression of the macrophage marker Mac2 in SMCs<sup>27</sup>. By contrast, neointima formation and monocyte counts were not altered in mildly hyperlipidemic Cxcr7<sup>−/−</sup> mice, indicating that the effect of CXCR7 on peripheral monocytes is indirect and dependent on the degree of hyperlipidemia. Concordantly, Cxcr7 deficiency further increased serum cholesterol levels only
in HCD-fed Apoe−/− mice, suggesting a regulatory role for CXCR7 in lipid metabolism under severe hyperlipidemic conditions. Apoe itself controls hematopoietic stem cell proliferation (by autonomously promoting cholesterol efflux via ABC-transporters), monocytosis, and lesional monocyte accumulation. However, it is unlikely that CXCR7 directly compensates for such effects, as it is not expressed on BM cells. Although Cxcr7 expression has been previously reported in lesional macrophages, we did not detect CXCR7 in injured arteries and genetic deletion of Cxcr7 in BM cells had no effect on neointima formation, indicating a minor role of Cxcr7 in macrophages during vascular repair.

Treatment of Apoe−/− mice with CCX771 led to the opposite phenotype to that expressed by Cxcr7−/− mice in terms of lesion formation, monocytosis, and hyperlipidemia; this indicates that CCX771 triggers the activation of CXCR7. Of note, CCX771 induces stronger β-arrestin-2 activation via CXCR7 than its canonical ligand, namely CXCL12. In line with previous reports, we found that CXCR7 is expressed in cells within the marginal zone of the spleen, most likely B cells, which are involved in protective immunity against atherosclerosis. However, removal of the spleen did not alter the effect of CCX771 on neointima formation and hyperlipidemia, suggesting that splenic CXCR7 expression does not play a role in atherosclerotic vascular disease. In contrast to neointima formation, both Cxcr7−/− and CCX771-treated mice showed increased CXCL12 levels in the plasma and BM, indicating that the endocytotic removal of CXCL12 is impaired by Cxcr7 deficiency as well as by CCX771 treatment. This parallel increase of CXCL12 levels in the plasma and BM maintained the gradient of CXCL12 between these two compartments, which prevents the mobilization of SPCs. However, this effect on extracellular CXCL12 levels cannot explain the contrary change of neointima formation in Cxcr7−/− and CCX771-treated mice.
The type of dyslipidemia observed in ApoE−/− mice is characterized by an elevation of VLDL/IDL-derived cholesterol levels, whereas LDL-cholesterol levels are only mildly increased and HDL-cholesterol levels are reduced. Treatment with CCX771 lowered only circulating VLDL levels in ApoE−/− mice by enhanced VLDL clearance and uptake to VAT, which is the primary storage site for unesterified cholesterol. Cholesterol uptake by adipocytes mainly occurs via triglyceride-rich lipoproteins, such as chylomicrons and VLDL. CCX771 treatment enhanced the uptake of cholesterol from VLDL to the VAT in a β-arrestin-2 dependent manner, which may explain the reduced serum cholesterol levels observed in CCX771-treated mice.

Moreover, CCX771 treatment enhanced lipase activity in adipose tissue presumably by down-regulating Angptl4, which inhibits the lipolytic activity of LPL by converting the active dimer into an inactive monomer. In addition to lipolysis, LPL dimers enhance the cellular uptake of VLDL independent of its catalytic activity by bridging between lipoproteins and lipoprotein receptors or proteoglycans. Increased LPL activity may cause the reduced VLDL cholesterol and TG serum levels in Angptl4−/− mice, and ApoE−/− mice lacking the Angptl4 gene develop less atherosclerosis. Conversely, overexpression of Angptl4 impairs the clearance of VLDL and results in hypertriglyceridemia and hypercholesterolemia in mice. Moreover, chronic intermittent hypoxia increases VLDL levels, reduces adipose LPL activity, and promotes atherosclerosis by up-regulating Angptl4 expression in adipose tissue of ApoE−/− mice. However, Angptl4 can also limit lipid accumulation in macrophages, which may play a role in the protection against atherosclerosis in mice overexpressing Angptl4. In summary, these data indicate that activation of CXCR7 under hyperlipidemic conditions lowers Angptl4 expression in adipose tissue and thereby reduces serum cholesterol levels through enhanced LPL-mediated uptake of VLDL to adipose tissue.
Taken together, these results suggest a novel role for the chemokine receptor CXCR7 in the uptake of VLDL into adipose tissue in Apoe−/− mice, which regulates serum cholesterol levels. Activation of CXCR7 by the synthetic ligand CCX771 protects against atherosclerosis, most likely by lowering serum lipid levels. Thus, treatment with CCX771 might be a promising therapeutic approach to treating atherogenic dyslipidemia.

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**Conflict of Interest Disclosures:** M.E.P. and T.J.S. are current employees of ChemoCentryx Inc. and have equity ownership on it.

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4. Tsou C-L, Peters W, Si Y, Slaymaker S, Aslanian AM, Weisberg SP, Mack M, Charo IF. Critical roles for CCR2 and MCP-3 in monocyte mobilization from bone marrow and


**Figure Legends:**

**Figure 1.** Cxcr7 deficiency increases neointimal macrophage accumulation. (A) Cxcr7 immunostaining in spleen sections of Apoe<sup>−/−</sup> mice in which Cxcr7 was deleted by activation of a ubiquitously expressed Cre-recombinase (CAG-<sup>+</sup>Cre<sup>+</sup>Cxcr7<sup>flax</sup>Apo<sup>−/−</sup> mice) 2 weeks after tamoxifen (TMX) treatment. TMX-treated Cxcr7<sup>flax</sup>Apo<sup>−/−</sup> mice not expressing Cre-recombinase were used as controls (CAG-<sup>+</sup>Cre<sup>+</sup>Cxcr7<sup>flax</sup>Apo<sup>−/−</sup> mice). Representative images are shown. Arrows indicate positive staining for Cxcr7. (B) Quantification of the neointimal and medial areas, and of the internal (IEL) and external elastic lamina (EEL) length 28 d after wire injury to the carotid arteries in CAG-<sup>+</sup>Cre<sup>+</sup>Cxcr7<sup>flax</sup>Apo<sup>−/−</sup> and CAG-<sup>+</sup>Cre<sup>+</sup>Cxcr7<sup>flax</sup>Apo<sup>−/−</sup> mice. n=8/group. (C) Quantification of neointimal macrophage numbers and of the Mac2 positive area was performed.
by Mac2 immunostaining (green) (n=6-8/group). (D) The neointimal SMC number and the
SMA$^+$ area were studied in the neointima using α-SMA immunostaining (green) (n=6-8/group).
Nuclei were counterstained with DAPI (blue). Scale bars, 200 μm (A) and 100 μm (B-D).
*P<0.05.

**Figure 2.** The effect of Cxcr7 deficiency on neointima formation is related to hyperlipidemia-induced monocytosis. (A) The circulating Sca-1$^+/Lin^-$ cell population was analyzed before and 24 h after vascular injury (n = 8–9/group). (B) CXCL12 levels were determined in the plasma (n = 6–7/group) and BM (n = 3/group) 28 d after vascular injury. (C) The expression level of Cxcr4 transcripts was quantified in various tissues of CAG-Cre$^+$Cxcr7$^{flox}$Apoe$^{-/-}$ and CAG-Cre$^+$Cxcr7$^{flox}$Apoe$^{-/-}$ mice (n = 3-6/group). BM=bone marrow; VAT=visceral adipose tissue; SM=skeletal muscle. (D) Neointima formation after carotid wire injury was quantified in TMX-treated CAG-Cre$^+$Cxcr7$^{flox}$Apoe$^{-/-}$ and CAG-Cre$^+$Cxcr7$^{flox}$Apoe$^{-/-}$ mice fed a HCD (n = 7–8/group). Serum cholesterol and triglyceride (TG) levels (E) and peripheral monocyte counts (F) were determined in TMX-treated CAG-Cre$^+$Cxcr7$^{flox}$ and CAG-Cre$^+$Cxcr7$^{flox}$ (either Apoe$^{-/-}$ or Apoe$^+$/-) after 28 d of HCD feeding (n = 5–10/group). (G) Fat, lean, and total weights of CAG-Cre$^+$Cxcr7$^{flox}$Apoe$^{-/-}$ and CAG-Cre$^+$Cxcr7$^{flox}$Apoe$^{-/-}$ mice were determined by micro-CT after 28 d of HCD feeding (n = 4/group). (H) In addition, fasting blood glucose and plasma insulin levels were measured and the quantitative insulin sensitivity check index (QUICKI) was determined in these mice (n = 4/group). *P<0.05; **P<0.01.

**Figure 3.** Treatment with the CXCR7 ligand, CCX771, reduces neointima formation in Apoe$^{-/-}$ mice. The effects of CCX771 treatment (compared with vehicle treatment) on neointima
formation, medial size (A and B), and the length of the IEL and EEL (A and C) after vascular injury to the carotid arteries of Apoe<sup>−/−</sup> mice were determined by planimetry (n=7–8/group). Macrophage numbers in the neointima were quantified by immunostaining of Mac2 (red; D and E). The relative SMC number and the neointimal SMA<sup>+</sup> area were measured by α-SMA staining (F) (n=7–8/group). Arrows delineate the neointima. Nuclei were counterstained with DAPI (blue). *P<0.05. Scale bars, 100 μm.

**Figure 4.** CCX771 ameliorates hyperlipidemia independent of the splenic Cxcr7 expression. The circulating Sca-1<sup>+</sup>/Lin<sup>−</sup> (A; n=8–9/group) and the CD34<sup>+</sup>/c-kit<sup>+</sup>/Lin<sup>−</sup> (B; n=4–5/group) cell population was determined before and 24 h after vascular injury. (C) CXCL12 plasma (n=6–7/group) and BM (n=3/group) levels were quantified 28 d after vascular injury. (D) Serum cholesterol and TG levels were determined in Apoe<sup>−/−</sup> mice fed a HCD and treated with CCX771 or vehicle 28 d after vascular injury (n=6/group). Quantitative analysis of circulating monocytes (E, n=6–7/group) and the ratio of Gr-1<sup>hi</sup> to Gr-1<sup>lo</sup> monocytes (F, n=4/group) in mice treated with CCX771 or vehicle was performed 28 d after vascular injury. (G and H) Splenectomized Apoe<sup>−/−</sup> mice fed a HCD were treated with vehicle or CCX771 for 28 d after vascular injury. The neointimal area in sections of the carotid artery (G; n=4/group) and serum cholesterol levels (H; n=3–4/group) was quantified. Representative images are shown. *P<0.05; **P<0.01; Scale bars, 200 μm.

**Figure 5.** CCX771 lowers VLDL cholesterol levels. Lipid metabolism was studied in Apoe<sup>−/−</sup> mice after 28 d of HCD feeding and treatment with CCX771 or vehicle. Fasting and non-fasting serum cholesterol (A) and TG (B) concentrations were studied (n=4–5/group). (C) Total serum

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cholesterol, VLDL-, LDL-, and HDL-cholesterol were quantified by FPLC (n=5-7/group). (D) The TG production rate was calculated from serum TG concentrations before and 6 h after treatment with P-407 (n=4/group). (E) The clearance of human ApoB100 and Dil was studied at 3 min (control) and 60 min after intravenous injection of Dil-VLDL (n=8-9/group). *P<0.05.

**Figure 6.** CCX771 enhances the uptake of VLDL into adipose tissue. Dil fluorescence intensity and cholesterol content in visceral adipose tissue (A; VAT) and in the liver (B) were determined 60 min after Dil-VLDL administration in Apoe⁻/⁻ mice fed a HCD and treated with CCX771 or vehicle for 28 d (n=4/group). (C) Dil-labeled VLDL (red) in epididymal adipose tissue from these mice was detected by in vivo multi-photon microscopy. (D) The Dil-VLDL uptake to adipocytes was studied in untreated (Blank), vehicle-, CCX704-, or CCX771-treated SGBS adipocytes. (E) Expression of β-arrestin2 (ARRB2) mRNA in SGBS adipocytes treated with siRNAs against ARRB2 or non-targeting siRNAs (ctrl-siRNA). (F) The effect of ARRB2 silencing on Dil-VLDL uptake to SGBS adipocytes treated with CCX771 or vehicle was determined. The data represent three independent experiments (D, E, and F). *P<0.05; ***P<0.001. Scale bars, 100 μm.

**Figure 7.** CCX771 increases lipase activity and inhibits Angptl4 expression in the VAT. Apoe⁻/⁻ mice were fed a HCD and treated with CCX771 or vehicle for 28 d. Lipase activity (A) and the protein concentration of Angptl4 (B) and Angptl3 (C) were measured in the plasma and VAT (n=4-5/group). (D) The mRNA expression levels of Lpl, Angptl4, Angptl3, and Pparg were quantified in the VAT (n=5-6/group). (E) Fasting and non-fasting blood glucose concentrations (E; n=4-6/group) and fasting plasma insulin levels were determined (F; n=3-4/group). (G) As a
surrogate marker for insulin sensitivity, the QUICKI was calculated (n=3-4/group). *P<0.05.

**Figure 8.** CCX771 treatment inhibits diet-induced atherosclerosis in mice by reducing hyperlipidemia and monocytosis. (A) The size of the atherosclerotic lesions in the aortic roots of Apoe<sup>−/−</sup> mice treated with vehicle or CCX771 was determined after 12 wk on a HCD. (B) Lipid accumulation was quantified by oil-red-O staining of en face-prepared thoracoabdominal aortas. (C) The macrophage number was studied in aortic root lesions by Mac2 immunostaining (red). (D) The number of SMCs and the SM22<sup>+</sup> area in the lesions was analyzed by SM22 staining. (E) Serum cholesterol and TG levels and peripheral monocyte counts were determined in Apoe<sup>−/−</sup> mice after 12 wk on a HCD treated with vehicle or CCX771. *P<0.05; **P<0.01; ***P<0.001; n=9-10/group; Scale bars, 200 μm.
Figure 1
Figure 2
Figure 3
Figure 4

A. Sca-1+Lin- cells (% of mononuclear cells) before and 24 h after treatment with Vehicle or CCX771.

B. CD34+ c-kit+Lin- cells (% of mononuclear cells) before and 24 h after treatment with Vehicle or CCX771.

C. Plasma CXCL12 (pg/ml) before and 24 h after treatment with Vehicle or CCX771.

D. Cholesterol (mmol/L) before and after treatment with Vehicle or CCX771.

E. TG (mmol/L) before and after treatment with Vehicle or CCX771.

F. Monocytes (cells/µL) before and after treatment with Vehicle or CCX771.

G. Neointimal area (x10^4 µm²) before and after treatment with Vehicle or CCX771.

H. Cholesterol (mmol/L) before and after treatment with Vehicle or CCX771.
Figure 5
Figure 6
Figure 7
Figure 8
Activation of CXCR7 Limits Atherosclerosis and Improves Hyperlipidemia by Increasing Cholesterol Uptake in Adipose Tissue
Supplemental Methods

X-gal staining

The Cxcr7 promoter activity was determined in Cxcr7^{+/LacZ} and Cxcr7^{+/+} mice by X-gal staining. After perfusion with 10 ml of 2% PFA, tissues were removed and post-fixed for 15 min in 2% PFA on ice, washed with PBS containing 2 mM MgCl₂, and then incubated in staining solution (5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM MgCl₂, 0.02% Nonidet P-40, 0.01% sodium deoxycholate, and 1 mg/ml X-gal [Life Technologies GmbH, Darmstadt, Germany; dissolved in dimethylformamide] in PBS) overnight at 37°C. Subsequently, the fixed tissues were frozen and embedded in Tissue-Tek® O.C.T. compound (Sakura Finetek Germany GmbH, Staufen, Germany) for cryosectioning. Cryosections were re-stained with the staining solution and nuclei were counterstained with Nuclear Fast Red (Vector Laboratories Inc., Burlingame, CA, USA).

Flow cytometry

To quantify murine Sca-1^{+}Lin^{-} cells, peripheral blood cells were treated with phycoerythrin (PE)-conjugated anti-mouse Sca-1 (Ly-6A/E) (clone D7, CA: 12-5981; eBioscience, Frankfurt, Germany) and the biotinylated anti-mouse hematopoietic lineage antibody panel (CD3, CD45R, CD11b, Ter-119, Ly-6G; eBioscience) followed by a Streptavidin-PE-Cy7 conjugate (eBioscience). Murine monocyte subtypes were studied by incubating peripheral blood cells with biotinylated anti-mouse CD3, CD45R, or Ter-119 antibodies (eBioscience), followed by incubation with a Streptavidin-PE-Cy7 conjugate (eBioscience), a PE-conjugated antibody against CD115 (Clone AFS98, eBioscience), or a Cy^{TM} 5.5-conjugated Gr-1 antibody (clone RB6-8C5, BD Biosciences, Heidelberg, Germany). To study Cxcr7 expression, cell suspensions were stained with a Cxcr7 antibody conjugated with PE-Cy7 (clone 8F11-M16; BioLegend, Fell, Germany). Cell suspensions derived from Cxcr7^{-/-} mice
and isotype control antibodies were used as negative controls. To measure murine Lin’Sca-1’CD34’CD117+ endothelial progenitor cells (EPCs)\(^1,2\), peripheral blood cells were incubated after erythrocyte lysis with AlexaFluor-647-conjugated anti-mouse CD34 (BD Biosciences), a PE-conjugated anti-mouse CD117 (BD Biosciences), and the biotinylated anti-mouse hematopoietic lineage antibody panel followed by a Streptavidin-PE-Cy7 conjugate.

Data were collected using a flow cytometer (FACSCanto™ II; BD Biosciences) and analyzed using FlowJo software (TreeStar, Inc., Ashland, USA).

**Blood chemistry and hematology**

Serum samples were analyzed by dry chemistry using a Vitros 250 Analyzer (Ortho Clinical Diagnostics GmbH, Neckargemünd, Germany) to determine the levels of cholesterol, triglycerides, creatinine, alanine transaminase (ALT) or aspartate transaminase (AST). The peripheral blood count was determined using a hematology analyzer and EDTA-anticoagulated blood (MEK-6450K, Nihon Kohden, Rosbach v.d.H., Germany).

**Quantitative RT-PCR (qRT-PCR)**

Total RNA was extracted from murine tissues using an RNeasy micro or mini kit (Qiagen) and reverse transcribed into cDNA using a Quantitect reverse transcription kit (Qiagen). Primer sets specific for murine Cxcr7, Cxcr4, Angptl3, Angptl4, Vldlr, Ldlr (all Quantitect primer assay; Qiagen), Lpl (sense 5’-AGAGAGAGGACTCGGAGACG-3’ and antisense 5’-AACTCAGGCAGGCCCTTTT-3’), and Pparg (sense 5’-TTCAGAAGTGCCCTTGCTGTG-3’ and antisense 5’-CCAACAGGCTTCTCCTTCTCG-3’) were used for PCR amplification (Quantitect SYBR green PCR kit; Qiagen) in a real time cycler (Opticon; Bio-Rad Laboratories GmbH, Munich, Germany). Gapdh, β-actin, and rpl13a were used as reference genes. Total RNA was extracted from human SGBS adipocytes using (mirVana miRNA}
isolation kit, Life technologies) and reverse transcribed using (High Capacity cDNA Reverse Transcription kit, Life technologies). The \textit{ARRB2} qRT-PCR assay was performed with a QuantiTect Primer assay and the QuantiTect SYBR green PCR kit (Qiagen) on a 7900HT RT-PCR system (Life technologies). Human GAPDH (sense 5’-CCATGGAGAAGGCTGGGGG-3’ and antisense 5’-CAAAAGTTGTCATGGATGACC-3’) was used as reference gene. Qbase\textsuperscript{PLUS} software was used for data analysis (Biogazelle NV, Zwijnaarde, Belgium).

\textbf{Micro-computed tomography (CT)}

\textit{CAG-Cre}^+\textit{Cxcr7}^{flox}\textit{Apoe}^{-/-} or \textit{CAG-Cre}^-\textit{Cxcr7}^{flox}\textit{Apoe}^{-/-} were scanned using dual energy micro-CT (TomoScope DUO, CT Imaging, Erlangen, Germany) before and 2 h after injection of an iodine-based contrast agent (eXIA™160XL, Binitio Biomedical Inc., Ontario, Canada). Subcutaneous (SAT), visceral (VAT), and brown adipose tissue (BAT) were interactively segmented using the Imalytics Preclinical software (Philips, Aachen, Germany). Abdominal VAT was discriminated from SAT by the abdominal muscles. An iodine map was generated for the segmentation of interscapular BAT according to the accumulation of the contrast agent. The fat mass was calculated from the fat volume using a density of 0.92 g/cm\textsuperscript{3}.

\textbf{Lipoprotein separation by FPLC and measurement of cholesterol levels}

Different lipoprotein fractions (VLDL, LDL, and HDL) were separated from the plasma using a Äkta FPLC system (GE Healthcare GmbH, Solingen, Germany) equipped with a Superose 6 10/300 column (GE Healthcare) and PBS as the running buffer. The cholesterol concentration in the lipoprotein fractions was determined using a cholesterol assay kit (Cayman Europe, Tallinn, Estonia).
Quantification of DiI and cholesterol concentration in murine tissues and plasma

*Apoe*−/− mice were fed a HCD and treated with CCX771 or vehicle (10% Captisol) for 4 wk. Human Dil-VLDL (50 µg/mouse, Kalen Biomedical, Montgomery Village, USA) was injected intravenously to fasting (5 h) mice 2 h after the last administration of CCX771 or vehicle. Blood samples were obtained at 3 and 60 min after the injection of Dil-VLDL. Moreover, VAT, BAT, liver, and skeletal muscle were harvested 60 min after injection of Dil-VLDL and perfusion with PBS. Tissues were homogenized in cell lysis buffer (Cell signaling Technology, Inc., Danvers, USA) using a TissueLyser LT homogenizer (Qiagen). The DiI fluorescence intensity was measured in the tissue homogenates (excitation wavelength: 514 nm; emission wavelength: 565 nm) and plasma using a microplate reader (Infinite M200, Tecan). A standard curve was generated using different concentrations of DiI-VLDL. The cholesterol concentration was determined after dissolving the lipids in assay buffer using a cholesterol assay kit (Cayman Europe). The DiI and the cholesterol concentration in the homogenates were normalized to the protein concentration in the samples.

Two-photon laser scanning microscopy (TPLSM)

Intact epididymal adipose tissue and carotid arteries were mounted in a perfusion chamber 60 min after the injection of Dil-VLDL (50 µg/mouse). The chamber was filled with Hanks’ balanced salt solution (Gibco® HBSS, Life Technologies, Inc.) and the temperature was constantly maintained at 37°C. The carotid arteries were perfused at a pressure of 60 mmHg and nuclei were fluorescently labeled (Syto41 Blue fluorescent nucleic acid stain, 1:1000 for 30 min, Invitrogen, Karlsruhe, Germany). Adipose tissues (AT) were embedded in agarose gels (3% agarose in HBSS) and covered with a coverslip (0.17 mm thick) and imaged using the Fluoview FV1000-MPE multiphoton system (Olympus Deutschland GmbH, Hamburg,
Germany) coupled with a Mai Tai® DeepSee™ HP-DS laser (Newport Spectra-Physics GmbH, Darmstadt, Germany). To determine the autofluorescence of the ATs, images were taken at 730 nm before dye injection. One hour after the i.v. injection of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate-labeled VLDL (DiI-VLDL; Kalen Biomedical, Montgomery Village, USA), the AT and the carotid arteries were perfused with PBS to remove DiI-VLDL from the vascular lumen. To detect the emitted fluorescent signals, two ultra-sensitive external non-descanned PMT detectors were tuned to the emission spectra of tissue autofluorescence (495–540 nm), Syto41 (454 nm), and the DiI dye (575–630 nm). Images (1024 × 1024 pixels) were obtained in the x–y plane using image acquisition software (FV10-ASW Ver3.0; Olympus).

ELISAs

Plasma was obtained from EDTA-anticoagulated blood after centrifugation (1000g, 10 min, at 4°C). The femurs were flushed with PBS (500 µl) and the perfusate collected. After centrifugation of the perfusate (300g for 5 min), the supernatant was used for analysis. The CXCL12 concentration was determined using a mouse CXCL12 ELISA kit (RayBiotech, Inc., Norcross, USA). The concentration of human ApoB-100 was determined in murine plasma samples following injection of human VLDL by an ELISA specific for human ApoB-100 (Demeditec diagnostics GmbH, Germany). Plasma and epididymal AT levels of Angptl3 and Angptl4 were measured using a mouse Angptl3 (Quantikine ELISA kit, R&D Systems, Wiesbaden, Germany) and a mouse Angptl4 (Cusabio Biotech, Wuhan, P. R. China) ELISA kit, respectively. Tissue levels of Angptl3 and Angptl4, and BM levels of CXCL12 were standardized to the protein concentration (DC Protein Assay Kit, Bio-Rad). Absorbance was measured at 450 nm using a plate reader (Infinite M200, Tecan Group Ltd., Männedorf, Switzerland).
Lipase activity

The lipase activity in the plasma and epididymal AT homogenates was determined in Apoe\(^{-/-}\) mice that were fed a HCD and treated with CCX771 or vehicle for 4 weeks using a lipase activity assay kit (Cayman Chemical Company, Ann Arbor, USA). Fluorescence intensities were measured every minute for 15 min at an excitation wavelength of 385 nm and an emission wavelength of 515 nm using a microplate reader (Tecan). The lipase activity in the AT was standardized to the protein concentration.

Hepatic triglyceride production rate

The hepatic TG production rate was determined following injection of Poloxamer 407 (P-407, a gift from BASF ChemTrade GmbH, Germany). Apoe\(^{-/-}\) mice were fed a HCD and treated with CCX771 or Captisol for 4 weeks. Following this treatment, P-407 (0.5 mg/g BW, IP) was injected to fasting mice. Blood samples were collected directly before and 6 hours after the injection of P-407. The plasma triglyceride concentration was determined using a triglyceride colorimetric assay kit (Cayman Europe, Tallinn, Estonia). The TG production rate was calculated from the difference in plasma TG concentrations over the 6 h interval following detergent injection and was expressed as µmol/kg BW/h using a plasma volume of 35 ml/kg BW.

Index of insulin sensitivity and resistance

The quantitative insulin sensitivity check index (QUICKI) was determined as a surrogate measure of insulin resistance. The QUICKI was calculated according the formula QUICKI = \(1/[\log(I_0) + \log(G_0)]\), where \(I_0\) is the fasting plasma insulin (µU/mL), and \(G_0\) is the fasting plasma glucose (mg/dl). Apoe\(^{-/-}\) mice were fed a HCD and treated daily with CCX771 or vehicle for 4 wk. Fasting (5 h) blood samples were collected 2 h after the last injection. To
assess postprandial glucose levels, non-fasting blood was drawn before the fasting period. In addition, \( CAG-Cre^+ \text{Cxcr}^\text{fl} \text{Apoe}^{+/+} \) and \( CAG-Cre^+ \text{Cxcr}^\text{fl} \text{Apoe}^{+/-} \) mice were fed a HCD for 28 d before fasting blood samples were obtained. The glucose and plasma insulin concentrations were determined using a glucometer (Contour® Blood Glucose Meter, Bayer Healthcare, Leverkusen, Germany) and an insulin ELISA kit (Crystal Chem, Inc., Chicago, Illinois, USA), respectively.

**Cell culture**

Human SGBS preadipocytes (courtesy provided by Dr. Wabitsch) were cultured and differentiated as previously described\(^8\). Briefly, SGBS preadipocytes were differentiated into adipocytes by incubation with serum- and albumin-free differentiation medium [DMEM/F12 medium (Life Technologies GmbH, Darmstadt, Germany) supplemented with 2 \( \mu \)M rosiglitazone, 25 nM dexamethasone, 100 nM cortisol, 0.01 mg/ml transferrin, 0.2 nM triiodothyronine, 250 \( \mu \)M IBMX, and 20 nM insulin (all from Sigma-Aldrich Chemie GmbH, Munich, Germany)] for 4 days followed by culturing in DMEM/F12 medium (Life Technologies) supplemented with 100 nM cortisol, 0.01 mg/ml transferrin, 0.2 nM triiodothyronine, and 20 nM insulin (all from Sigma-Aldrich) for 10 d. The adipocyte differentiation was confirmed by Oil red O staining. The uptake of Dil-VLDL by SGBS adipocytes was studied after incubation with Dil-VLDL (5 \( \mu \)g/ml) for 2 h and pretreatment with CCX771 (1 \( \mu \)M in 10% DMSO), CCX704 (1 \( \mu \)M, an analog of CCX771 with low affinity for CXCR7), or vehicle (10% DMSO). In some experiments, SGBS adipocytes were transfected with siRNAs against human \textit{ARRB2} or a non-targeting siRNA (1 \( \mu \)M, Accell SMARTpool siRNA in Accell delivery cell culture medium, Dharamacon, Fisher Scientific – Germany GmbH, Schwerte, Germany) after 3 d in culture. After the incubation with Dil-VLDL, cells were washed 3 times with PBS (containing 1% bovine serum albumin) and Dil
was extracted with isopropanol. After centrifugation (1000g, 15 min), the DiI fluorescence intensity was measured in the supernatant using a microplate reader (Tecan).

To detect CXCR7 expression, SGBS adipocytes cultured on Poly-D-Lysine-coated coverslips (BD Biosciences) in 24-well plates (Corning B.V. Life Sciences, Amsterdam, The Netherlands) were fixed with 2% PFA and, following cooking in citrate buffer, incubated with a CXCR7 antibody (clone 11G8). A secondary Cy3-conjugated anti-mouse antibody (1:300, Jackson ImmunoResearch Europe Ltd., Suffolk, UK) was used to detect the primary antibody.
Supplemental Figure 1: Expression of *Cxcr7* mRNA in different tissues and *Cxcr7* protein on hematopoietic cells and ECs. *Cxcr7* (A) and *Cxcr4* (B) mRNA expression in various murine tissues was quantified by qRT-PCR (n = 3/group). (C) Flow cytometric analysis of CXCR7 expression on leukocytes, BM cells, and platelets from *Cxcr7*+/+ (open histogram) and *Cxcr7*−/− mice (grey shaded histogram) (n = 3/group). The expression of CXCR7 on activated (open histogram) and unstimulated (dotted line histogram) SVECs was studied. SVECs in the negative control group (grey shaded histogram) were incubated with an isotype control antibody. *P*<0.0001 versus carotid artery.
Supplemental Figure 2: Cxcr7 promoter activity in mice. Cxcr7 promoter activity was determined by X-gal staining of different tissues from Cxcr7<sup>+/LacZ</sup> and Cxcr7<sup>+/+</sup> mice (n = 3). Representative images are shown. Scale bars, 100 µm.
Supplemental Figure 3: Cxcr7 protein expression in mice. Immunostaining of Cxcr7 (green) in the spleen, aortic root, and kidney of Cxcr7⁻/⁻ and Cxcr7⁺/⁺ mice (n = 3). Nuclei were counterstained with DAPI (blue). The white rectangles indicate the area shown in a higher magnification in the middle column. Scale bars, 200 µm.
Supplemental Figure 4: CXCR7 expression is absent in normal and injured carotid arteries. (A) Carotid arteries of Cxcr7+/+ and Cxcr7−/− mice were immunostained for CXCR7. (B) CXCR7 immunostaining was performed in carotid artery sections of Apoe−/− mice fed a HCD 28 d after wire injury. An isotype control antibody (IgG) was used for negative control staining. Nuclei were counterstained with DAPI. Scale bars, 50 µm.
Supplemental Figure 5: Effect of Cxcr7 deficiency in BM cells on neointima formation and hyperlipidemia Apoe−/− mice harboring CAG-CreCxcr7<sup>flox</sup>Apoe<sup>−/−</sup> or CAG-Cre<sup>+</sup>Cxcr7<sup>flox</sup>Apoe<sup>−/−</sup> BM cells were subjected to carotid wire injury and fed a HCD for 28 d. The neointimal area was determined in sections of the carotid arteries (A). Serum cholesterol and TG levels (B), and the peripheral monocyte counts (C) were quantified in the blood. n = 4/group. Scale bars, 100 µm.
Supplemental Figure 6: Role of Cxcr7 deficiency in adipose tissue distribution. CAG-Cre\(^{-}\) Cxcr7\(^{\text{flo}x}\) Apoe\(^{-/-}\) and CAG-Cre\(^{+}\) Cxcr7\(^{\text{flo}x}\) Apoe\(^{-/-}\) mice were studied by micro-CT after 28 days of HCD feeding. (A) Subcutaneous (SAT) and VAT volumes were quantified in native micro-CT scans of the whole body. VAT mass relative to total white adipose tissue (WAT) mass, including VAT and SAT, was determined. (B) The interscapular BAT volumes were determined after injection of a contrast agent. Absolute BAT mass and BAT mass normalized to the lean BW were analyzed. The weights of the adipose tissues were calculated from the volumes using a density of adipose fat of 0.92 g/cm\(^3\). n = 3-4/group. Representative 3D reconstructions are shown.
Supplemental Figure 7: Pharmacology of CCX771. (A) The plasma concentration of CCX771 was determined in Apoe\textsuperscript{-/-} mice at 1 h and 12 h after s.c. injection of CCX771 (n = 9/group). AST (B) and Creatinine (C) levels in the serum of Apoe\textsuperscript{-/-} mice were quantified after 28 d of treatment with CCX771 or vehicle (n = 6–7/group). *P<0.05 versus 12 h.
Supplemental Figure 8: CXCL12 levels in Cxcr7⁻/⁻ mice following treatment with CCX771. The CXCL12 protein concentrations in the plasma and BM were determined in Cre⁺ Cxcr7flx Apoε⁻/⁻ mice fed a HCD and treated with CCX771 or vehicle for 28 d. n = 4-5/group.
Supplemental Figure 9: Effect of CCX771 on serum lipid levels in \(Ldlr^{-/-}\) mice. \(Ldlr^{-/-}\) mice were fed a HCD and treated with CCX771 or vehicle for 28 d before cholesterol and TG levels were analyzed in the serum. \(n = 4-5/group\). *\(P<0.05\).
Supplemental Figure 10: Role of CCX771 in TG production. Apoe<sup>−/−</sup> mice were fed a HCD and treated with CCX771 or vehicle for 28 d. Fasting serum TG levels were determined before (0 h) and 6 h after injection of P-407. n = 4/group. *P<0.05.
Supplemental Figure 11: CCX771 does not increase the uptake of VLDL to BAT and skeletal muscle. *Apoe*<sup>−/−</sup> mice were fed a HCD and treated with CCX771 or vehicle for 28 d before Dil-VLDL was injected intravenously. The DiI fluorescence intensity was quantified 60 min after the administration of Dil-VLDL in the BAT and skeletal muscle. n = 4-5/group.
Supplemental Figure 12: The role of Cxcr7 in the uptake of VLDL to the arterial wall.

(A) The uptake of Dil-VLDL to carotid arteries of Apoe<sup>−/−</sup> mice after feeding a HCD and treatment with CCX771 or vehicle for 28 d was studied 60 min after the injection of Dil-VLDL by multi-photon microscopy. Nuclei were counterstained with Syto41 (blue). Green = elastin; red = Dil. (B) The accumulation of Dil-VLDL to the carotid artery wall was analyzed in histological sections from CAG-Cre<sup>−</sup>Cxcr7<sup>flx</sup>Apoe<sup>−/−</sup> and CAG-Cre<sup>+</sup>Cxcr7<sup>flx</sup>Apoe<sup>−/−</sup> mice by epifluorescence microscopy. Nuclei were counterstained with DAPI (blue).
Supplemental Figure 13: CXCR7 protein expression in adipocytes. SGBS adipocytes were stained with an antibody against CXCR7 or an isotype control antibody (Ctrl IgG). Nuclei were counterstained with DAPI. Scale bars, 25 µm.
Supplemental Figure 14: Expression of *Vldlr*, *Ldlr*, and *Cxcr7* in adipose tissue. (A) Expression of *Vldlr* and *Ldlr* mRNA in epididymal adipose tissues from *Apoe<sup>−/−</sup>* mice fed a HCD and treated with vehicle or CCX771 for 28 d was determined by qRT-PCR (n = 3/group). (B) *Cxcr7* and *Cxcr4* mRNA expression in various visceral adipose tissues (AT) and spleen (n = 3/group). *P*<0.05 versus spleen.
Supplemental Figure 15: Renal and hepatic toxicities of long-term CCX771 treatment.

(A) Creatinine levels in the serum of Apoe<sup>−/−</sup> mice were quantified after 12 wk of treatment
with CCX771 or vehicle (n = 10/group). (B) GPT/ALT serum levels in Apoe<sup>−/−</sup> mice were
determined after 12 wk of treatment with CCX771 or vehicle (n = 10/group).
Supplemental References


