CXCL12 promotes atherosclerosis by downregulating ABCA1 expression via the CXCR4/GSK3β/β-cateninT120/TCF21 pathway

Running Title: CXCL12 aggravates atherosclerosis

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Abbreviations: LV, lentiviral vectors; CXCL12, CXC chemokine ligand 12; CXCR4, CXC chemokine receptor 4; MPM, mouse peritoneal macrophage; CAD, coronary artery disease; ABCA1, ATP binding cassette transporter A1; RCT, reverse cholesterol transport; HDL-C, high-density lipoprotein cholesterol; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglyceride; CE, cholesterol ester; apoA-I, apolipoprotein A-I; GSK3β, Glycogen synthase kinase 3β; Thr, threonine; Tyr, tyrosine; TCF21, transcription factor 21; EC,
endothelial cell; VSMC, smooth muscle cell;
Abstract

CXC chemokine ligand 12 (CXCL12) is a member of CXC chemokine family and mainly acts on cell chemotaxis. CXCL12 also elicits a pro-atherogenic role, but the molecular mechanisms have not been fully defined yet. We aimed to reveal if and how CXCL12 promoted atherosclerosis via regulating lipid metabolism. In vitro, our data showed that CXCL12 could reduce ABCA1 expression and it-mediated cholesterol efflux from THP-1-derived macrophages to apoA-I. Data from the Luciferase reporter gene and Chromatin immunoprecipitation assays revealed that TCF21 stimulated transcription of ABCA1 via binding to its promoter region, which was repressed by CXCL12. We found that CXCL12 increased the levels of phosphorylated GSK3β and phosphorylation of β-catenin at the position Thr120. Inactivation of GSK3β or β-catenin increased expression of TCF21 and ABCA1. Further, knockdown or inhibition of CXCR4 (CXC chemokine receptor 4) blocked the effects of CXCL12 on TCF21 and ABCA1 expression and phosphorylation of GSK3β and β-catenin. In vivo, overexpression of CXCL12 in Apoe-/- mice via lentivirus enlarged atherosclerotic lesion area and increased macrophage infiltration in atherosclerotic plaques. We further found that overexpression of CXCL12 reduced the efficiency of reverse cholesterol transport and plasma HDL-C levels, decreased ABCA1 expression in aorta and mouse peritoneal macrophages (MPMs), and suppressed cholesterol efflux from MPMs to apoA-I in Apoe-/- mice. Collectively, these findings suggest that CXCL12 interacts with CXCR4 and then activates the GSK-3β/β-cateninT120/TCF21 signaling pathway to inhibit ABCA1-dependent cholesterol efflux from macrophages and aggravate atherosclerosis. Targeting CXCL12 may be a novel and promising strategy for the prevention and treatment of atherosclerotic cardiovascular diseases.

Key Word: CXCL12, atherosclerosis, CXCR4, ABCA1, cholesterol efflux
1. Introduction

Atherosclerosis is a chronic vascular disease that is identified as one of the pathogenesis of cardiovascular disease. It is well known that atherosclerosis is driven by dysregulation of cholesterol metabolism that leads to the formation of foam cell, a hallmark of atherosclerosis(1). Reverse cholesterol transport (RCT) is an important approach to prevent lipid accumulation during atherogenesis(2, 3). ATP binding cassette transporter A1 (ABCA1) locates in plasma membrane, serving as a cholesterol transporter to mediate excessive cholesterol efflux from macrophages to apolipoprotein A-I (apoA-I). This is believed to be the first and the most important step of RCT. Indeed, several lines of evidence demonstrated that ABCA1 expression and cholesterol efflux are inversely associated with the development and progression of atherosclerosis(4-7). Thus, understanding how ABCA1 expression is regulated has always been a hot research topic.

Chemokine is a group of molecules that mainly function on cell chemotaxis and are extensively expressed in vascular cells, such as macrophages, endothelial cells (ECs), and smooth muscle cells (SMCs). CXCL12 belongs to the CXC-subclass of the chemokine family. Several clinical reports showed that higher serum levels of CXCL12 are closely related to the increase in atherosclerotic risk (8-11). Overexpression of CXCL12 can aggravate atherosclerosis progression in Apoe-/-- mice(12, 13). CXCR4, a specific receptor for CXCL12, is strongly correlated with plaque stability, lesion area, and risk for cardiovascular disease (14-16). Merckelbach et al reported that CXCL12 and CXCR4 were abundantly expressed in macrophages within human carotid artery atherosclerosis(17). It has been shown that CXCL12 stimulates the formation of macrophage-derived foam cell via CXCR4 (18). However, the role of CXCL12 in cholesterol efflux is unclear.

Glycogen synthase kinase 3β (GSK3β) is a multifunctional serine/threonine kinase, mainly involving in cellular glucose metabolism(19). Activation of GSK3β accelerates, while its knockdown inhibits atherosclerosis(20, 21). Although GSK3β has been implicated in dyslipidemia during atherogenesis, whereas the role of GSK3β in ABCA1-mediated cholesterol efflux has yet to be determined. β-catenin belongs to the catenin family and commonly acts as a nuclear transcriptional activator to activate transcription of target genes when Wnt/β-catenin is activated (22). GSK3β-mediated change in protein phosphorylation regulates translocation of β-catenin from cytoplasm to nucleus and the subsequent GSK3β/β-catenin pathway. This has been
implicated in the development of cardiovascular disease (23-26). Transcription factor 21 (TCF21) is a member of the basic helix-loop-helix (bHLH) transcription factor (TCF) family and affects the development of coronary vasculature (27). Gu et al. found that TCF21 was associated with the risk of coronary artery disease (CAD) in Chinese Han population (28). Inhibition of TCF21 expression dramatically increases CAD risk (29). However, the underlying mechanisms remain to be addressed.

In this study, we found that binding of CXCL12 to CXCR4 activated the GSK3β/β-cateninT120/TCF21 pathway. This downregulated ABCA1 expression and reduced cholesterol efflux. Further, our data confirmed the pro-atherogenic role of CXCL12 in Apoe-/- mice.
2. Materials and methods

2.1 Animals

Apoe-/- male mice on the C57BL/6 background (8 weeks old) were purchased from the Department of Laboratory Animal of Cavens (ChangZhou, China). Apoe-/- mice were injected with 1 x 10^{11} viral particles of LV-CXCL12 or LV via the tail vein and then fed the Western-type diet for 12 weeks. After, mice were euthanized using pentobarbital sodium. Blood samples and tissues were collected. Prior to euthanasia, mice were intraperitoneally injected with 4% thioleicollate broth, followed by injection of 5 ml PBS for collection of peritoneal macrophages. All procedures were done in line with the Institutional Animal Ethics Committee and the University of South China Animal Care Guidelines for the Use of Experimental Animals.

2.2 Atherosclerosis analysis

Aorta was dissected from mice with all adventitia removed. Quantification of lesion size and composition is in agreement with the recommendation of ATVB guideline and the previous studies (30, 31). Aortas were then unfolded along the longitudinal axis, stained with Oil Red O, and photographed with a CASIO EX-ZR3700 digital camera en face to measure the percentage of total atherosclerotic lesions on the aortic surface. Mouse hearts were sectioned perpendicular to the axis of the aorta, and once the aortic root was identified by the appearance of aortic valve leaflets. 8 serial sections (10 µm intervals) of aortic sinus were obtained per mouse. We randomly selected 3 sections from each mouse per group for Oil Red O, HE and Masson staining. The lesion areas were quantified with Oil Red O staining, and the collagen contents were quantified with Masson staining. All analyses were quantified using Image-J software.

2.3 Plasma lipids profiles

Plasma lipid levels were analyzed as previously described(7). Blood samples were collected from the retro-orbital plexus of Apoe-/- mice injected with or without LV-CXCL12 or saline and fed the Western-type diet for 12 weeks. Plasma levels of total cholesterol (TC), LDL cholesterol (LDL-C), HDL-cholesterol (HDL-C), and triglyceride (TG) levels were analyzed by enzymatic methods using their specific kits (BioSino Bio-Technoleogy and Science Inc, China).
2.4 Immunofluorescence assay

The sections of aortic roots were washed with 1X PBS three times and blocked with 5% goat serum for 30 min at room temperature. THP-1-derived macrophages were incubated with or without CXCL12, washed with PBS, and then fixed with methanol. After, the sections and cells were incubated with anti-ABCA1 antibody (mouse monoclonal antibody, 1:200, Abcam, U.K.), anti-CD68 antibody (mouse monoclonal antibody, 1:200, Abcam, U.K.), or anti-CXCR4 antibody (mouse monoclonal antibody, 1:200, Abcam, U.K.) overnight at 4°C, followed by Cy3-labeled goat anti-mouse IgG (H+L) (1:200, Beyotime, China) for 1 h at room temperature in a dark place. Nuclei were counterstained with DAPI (Thermo Scientific, China). Fluorescence microscopy of ABCA1 and CD68 in the sections, and ABCA1 and CXCR4 in cells were performed using an EVOS FL AUTO 2 (Thermo Scientific, China). The mean fluorescence intensity (MFI) on the stained sections of aortic roots and cells were quantified using Image J software.

2.5 Immunohistochemistry

Mice aorta root sections were incubated with UV block (Thermo Scientific-Pierce) containing 10% goat serum (ABCAM, USA) for 30 min and then with mouse monoclonal anti-CD68 antibody (ABCAM, 1:1000) overnight in a humid chamber at 4 °C. After, sections were incubated with a biotin-conjugated secondary antibody for 1 h at 37 °C, followed by streptavidin-HRP for 30 min. Sections were then counterstained with hematoxylin for 15 s, differentiated with a hydrochloric acid/alcohol mixture, and then the DAB stain for 30 s. After washing with water for 15 min, sections were imaged using an EVOS FL AUTO 2 (Thermo Scientific, China).

2.6 Measurement of RCT in vivo

The efficiency of RCT was determined as previously described(7). J774.1 macrophages were loaded with 50 μg/mL ac-LDL and 5 μCi/mL [3H] cholesterol for 24 h in DMEM. The labeled J774 cells were injected into the abdominal cavity of individual mouse. Plasma was collected at 6, 24, and 48 h after injection. The feces were continuously collected from 0-48 h until the endpoint of the experiment. The feces were weighed and dissolved in 50% ethanol. 20 μL of aliquots were used for scintillation counting after shaking overnight. The liver was collected from euthanized mice, washed in ice-cold PBS, blotted up with filter papers, weighted, and stored at -20°C. 80 mg
of frozen liver tissue was added into n-hexane and isopropanol with a proportion of 3:2 with shaking for 10 min. After, the samples were vacuum dried for the extraction of liver lipids and radioactivity was counted with a liquid scintillation counter. The results were calculated as the percentage of injected = CPM (plasma, liver or feces) / total CPM (5 μCi/mL [3H] cholesterol).

2.7 Cell culture and transfection

Human THP-1 monocytes were purchased from Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI-1640 supplemented with 0.1% nonessential amino acids, penicillin (100 U/mL), streptomycin (100 mg/mL) and 10% FBS. HEK293T cells were purchased from the CAS Cell Bank and cultured in DMEM containing 10% FBS. Cells were incubated at 37°C in a humidified atmosphere of 5% CO2. The differentiation of THP-1 monocytes into macrophages was induced by 160 nM phorbol-12-myristate acetate (PMA) for 24 h.

THP-1-derived macrophages and HEK293T cells were cultured in 12-well plates for different treatments. β-catenin agonist SKL2001 (MedChemExpress, USA), GSK3β inhibitor TWS119 (MedChemExpress, USA), CXCR4 inhibitor LY2510924 (MedChemExpress, USA), or recombinant human CXCL12 protein (ABCAM, USA) was incubated with cells. CXCR4 siRNA (ABCAM, USA) and TCF21 (Santa Cruz Biotechnology, USA) were introduced to cells by transfection and lentivirus vector respectively. 6–12 h later, medium was changed to DMEM containing antibiotics and 10% FBS.

2.8 Western blot analysis

Tissues and cells-derived total proteins were extracted by the regular method. Nuclear and cytoplasmic proteins were extracted by nuclear and cytoplasmic protein extraction kit (Sangon Biotech, China). β-actin was used as the loading control for total proteins derived from tissues and cells, and cytoplasmic proteins. H3 was used as the loading control for nuclear fraction (32). Total proteins were quantified and subjected to SDS-PAGE, followed by immunoblotting. Primary antibodies included anti-GSK3β (Santa Cruz Biotechnology, 1:1000), anti-phospho-GSK3β (Santa Cruz Biotechnology, 1:1000), anti-β-catenin (Santa Cruz Biotechnology, 1:1000), anti-phospho-β-cateninY654 (ABCAM, 1:1000), anti-phospho-β-cateninT120 (Santa Cruz Biotechnology, 1:2000), anti-CXCR4 (Santa Cruz Biotechnology, 1:200), anti-ABCA1 (Santa
Cruz Biotechnology, 1:200), anti-TCF21 (Santa Cruz Biotechnology, 1:10000), anti-Histone H3 (Santa Cruz Biotechnology, 1:500), and anti-β-actin (Santa Cruz Biotechnology, 1:2000).

Secondary antibodies were HRP-labelled Goat anti-Mouse IgG (H + L) (Santa Cruz Biotechnology, 1:1000). Antibody binding was visualized with Tanon 5500 (China) and BeyoECL Plus (Beyotime, China).

2.9 Co-immunoprecipitation

THP-1-derived macrophages were incubated with CXCL12 and then homogenized and lysed in RIPA buffer. Protein A plus-agarose (Thermo Fisher, USA) were washed in PBS three times. Lysates were incubated with antibodies overnight at 4 °C, followed by an incubation with pre-washed protein A plus-agarose overnight at 4 °C. Beads were then washed three times in PBS. Immunoprecipitated proteins were eluted from the bead with 60 μL of 2 X sodium lauryl sulfate (SDS) sample buffer (Beyotime, China) containing 2-mercaptoethanol (2ME) at 60 °C for 5 min, and then subjected to immunoblotting.

2.10 Cholesterol efflux assay

Cholesterol efflux was performed as described previously (4). THP-1-derived macrophages were incubated with or without CXCL12 for 12 h and then cultured in 0.1% BSA and RPMI 1640 medium for 24 h. Peritoneal macrophages were isolated from Apoe-/- mice and cultured in RPMI 1640 medium containing 0.1% BSA for 24 h. Mouse peritoneal macrophages and THP-1-derived macrophages were incubated with 50 μg/ml ox-LDL and then labeled with 0.5 μCi/ml [3]H-cholesterol for 24 h in serum-free medium. After, the cells were washed in PBS and then cultured in RPMI 1640 medium containing 0.1% BSA and 25 μg/ml apoA-I or 50 μg/ml HDL (Sigma-Aldrich, U.S.A) for 24 h. Radioactivity in culture medium and cells were counted in a liquid scintillation counter (LSC) separately. Cholesterol efflux was calculated as the ratio of [3]H-cholesterol in medium to total [3]H-cholesterol in cells and medium.

2.11 High-performance liquid chromatography (HPLC) analysis of lipids

The lipid content in THP-1-derived macrophages was examined as previously described(4).
THP-1-derived macrophages were washed three times with PBS. The cells were homogenized in 1 ml of 0.9% NaCl on ice using the ultrasonic processor (Cole Parmer, U.S.A). Protein concentrations were measured using BCA Protein Assay kit (Abcam, China). Isopropanol (1 mg cholesterol/ml) was added to extract cholesterol and then stored at 20℃. The stock solution was diluted as cholesterol standard liquid and then supplied with 10 μl of reaction mixture containing 500 mM MgCl₂, 500 mM Tris-HCl (pH7.4), 10 mM dithiothreitol, and 5% NaCl to 0.1 ml cholesterol standard liquid. After, 0.4 U cholesterol oxidase in 10 μl of 0.5% NaCl was added into each sample for examination of free cholesterol or 0.4 U cholesterol oxidase together with 0.4 U cholesterol esterase for measurement of total cholesterol. Sample were incubated at 37℃ for 30 min. The reaction was then terminated by addition of 100 ml of methanol: ethanol (1:1). After cooling for 30 min, samples were centrifuged at 1500 rpm for 10 min at 15℃. 10 μl of supernatant was applied to a 2790 Chromatographer (Waters, U.S.A) for chromatographic analysis. Absorbance at 216 nm was monitored with cholesterol content indicated by the peak area.

2.12 Real-time quantitative PCR

THP-1-derived macrophages, mouse peritoneal macrophages (MPMs) and tissues were lysed for RNA extraction using TRIzol. The sequences of the real-time PCR primers were: Human ABCA1, 5'-GTCTCTTTGCCATTATCTGG-3' and 5'-CCTCCTCCTCGC TCGCAAT-3'; Human TCF21, 5'-GGTTAGTTAGGAGGGGAAGTA-3' and 5'-ACACCCAAAACAAAATAATCTTA-3'; Mouse ABCA1: 5'-GGGTGGGTCTCTACAC-3' and 5'-GAATGACGAGGATGAGGATGTG-3'. Analysis of mRNA levels was performed on an ABI PRISM 7900 sequence detection system (Applied Biosystems).

2.13 Luciferase reporter gene

The TCF21 expression vector was acquired from Genechem (Shanghai, China). The luciferase vectors with the promoter region of 3000bp (pro 3000) of the human ABCA1 were prepared by Genechem. HEK293T cells were treated with CXCL12 or transfected with 0.5 μg of TCF21 overexpression vector using Lipofectamine 2000 (Invitrogen, U.S.A). The cells were then co-transfected with 0.5 μg of ABCA1 promoter reporter construct and renilla luciferase control reporter vector. After 12 h, cells were washed in PBS and incubated in medium containing 0.1%
BSA and RPMI 1640. The luciferase activity was detected using the Dual-Glo Luciferase Assay System (Promega, USA). The results were standardized in the corresponding luciferase activity and plotted as a percentage of the control.

2.14 Chromatin immunoprecipitation assay

THP-1-derived macrophages transfected with CXCL12 were cross-linked in 1% formaldehyde for 15min at 37°C. The reaction was stopped by adding glycine solution. SDS Lysis Buffer (Beyotime, China) and phenyl methyl sulfonyl fluoride (PMSF) (Beyotime, China) were then added to the cells before sonication with an ultrasonic processor (Sonics, U.S.A) for 14 bursts of 4.5s with 9s intervals under 60W on the ice. Cell lysate was centrifuged at 12000 rpm for 10min at 4°C. The supernatant containing sheared chromatin was kept on ice. DNA fragment sizes were measured using agarose gel electrophoresis. After, the samples were subjected to immunoprecipitation using a CHIP assay kit (ABCAM, U.K.) and antibody against TCF21 (ABCAM, U.S.A) or antibodies against IgG (ABCAM, U.S.A). The DNA was eluted and collected for analysis of quantitative real-time PCR using following primers. Human ABCA1 Primers: Forward 5′-CTCGGTGCAGCCGAATCTAT-3′ and Reverse 5′-CACTCACTCTCGCTCGCAAT-3′.

2.15 Statistical analysis

All data were presented as means ± SD. The distribution of all data was analyzed by F-test and showed no significant difference ($P > 0.05$). Statistical significance was evaluated by either one-way ANOVA or Student’s t-test. Student’s t-test was used for assessing the differences between two groups, while one-way ANOVA is used for analyzing the difference between multiple groups. Scheffe’s test was employed for specific comparisons. A value of $P < 0.05$ was considered as statistical significance.
3. Results

3.1 CXCL12 downregulates ABCA1 expression and inhibits cholesterol efflux in THP-1-derived macrophages.

To investigate the role of CXCL12 in lipid metabolism, we treated THP-1-derived macrophages with CXCL12. As shown in Fig. 1A, CXCL12 reduced the mRNA and protein levels of ABCA1. To confirm this finding, we detected ABCA1 in THP-1-derived macrophages using immunofluorescence microscopy. Consistently, cellular ABCA1 signal was markedly reduced in CXCL12-treated cells (Fig. 1B). Given the important role of ABCA1 in the maintenance of cellular cholesterol homeostasis, we measured cholesterol efflux onto apoA-I and HDL and found that CXCL12 reduced cholesterol efflux to apoA-I but not to HDL (Fig. 1C). Furthermore, CXCL12 significantly increased cellular content of total cholesterol (TC), triglyceride (TG), and cholesterol ester (CE) in THP-1-derived macrophages (Table 1). Together, these findings indicate that CXCL12 exerts an inhibitory effect on cholesterol efflux from THP-1-derived macrophages via reducing ABCA1 expression.

3.2 TCF21 plays an important role in CXCL12-induced reduction in ABCA1 expression.

TCF21 binds to the promoter region of its target and regulates their expression. We explored the promoter activity of ABCA1 in response to TCF21 and CXCL12 in HEK293T cell using the luciferase assay and found that TCF21 enhanced while CXCL12 decreased ABCA1 promoter activity (Fig. 2A-B). Consistently, results obtained from the chromatin immunoprecipitation (CHIP) assay indicated that CXCL12 markedly inhibited binding of TCF21 to ABCA1 gene in THP-1 derived macrophages (Fig. 2C). Next, we assessed the effect of CXCL12 on TCF21 and ABCA1 expression in THP-1 derived macrophages. As shown in Fig. 2D-E, CXCL12 significantly decreased the mRNA and protein levels of TCF21 and inhibited the TCF21-induced upregulation of ABCA1 expression. These results suggest that CXCL12 inhibits TCF21 expression, decreasing ABCA1 transcription and consequently downregulating its expression.

3.3 CXCL12-induced reduction in ABCA1 expression is mediated by GSK3β/β-cateninβ20.

Given that β-catenin nuclear translocation regulates the transcriptional activation of TCF family members, we hypothesized that β-catenin might act as an upstream molecule for the
CXCL12-regulated expression of ABCA1. To test it, we evaluated the protein levels of β-catenin in THP-1-derived macrophages. β-actin and Histone 3 (H3) were used as the loading control for cytoplasmic and nuclear fraction, respectively. Our results indicated that treatment of CXCL12 strikingly decreased the amount of nuclear β-catenin but increased cytoplasmic β-catenin (Fig. 3A). Additionally, the expression of TCF21 and ABCA1 were significantly upregulated by a β-catenin agonist, SKL2001 (Fig. 3B-C). Because the cellular localization of β-catenin is regulated by its phosphorylation, we examined phosphorylated β-catenin using two antibodies that can specifically recognize phosphorylation at positions Thr120 and Tyr654. As shown in Fig. 3D, CXCL12 increased levels of p-β-catenin\textsubscript{T120} but not p-β-catenin\textsubscript{Y654}. Considering that phosphorylation of GSK3β reduced nuclear β-catenin, we assessed the contribution of GSK3β to CXCL12-reduced ABCA1 expression. Indeed, CXCL12 increased the level of p-GSK3β (Fig. 4A). Furthermore, GSK3β inhibitor, TWS119, partially blocked CXCL12-induced cytoplasmic retention of β-catenin and the levels of phosphorylated β-catenin\textsubscript{T120} (Fig. 4B-C). In addition, TWS199 markedly upregulated expression of TCF21 and ABCA1 (Fig. 4D-E). Taken together, these data suggest that the GSK3β/β-catenin\textsubscript{T120} pathway acts the upstream signal for the inhibitory effect of CXCL12 on the expression of TCF21 and ABCA1.

**3.4 CXCR4 is essential for the effect of CXCL12 on ABCA1 expression.**

CXCR4 is a specific receptor for CXCL12 and involves multiple CXCL12-induced signal transduction. We performed co-immunoprecipitation and observed that CXCL12 indeed interacted with CXCR4 in THP-1 macrophages (Fig. 5A). To analyze the knockdown efficiency of CXCR4 siRNA, we examined the protein levels and fluorescence intensity of CXCR4 in THP-1 derived macrophages using Western blot and immunofluorescence assay, respectively. As shown in Fig. 5B-C, expression and fluorescence intensity of CXCR4 were efficiently reduced in THP-1-derived macrophages transfected with CXCR4 siRNA. In addition, we found that the effect of CXCL12 on phosphorylation of GSK3β was blocked by knockdown of CXCR4 and a CXCR4 inhibitor, LY2510924 (Fig. 5D). We then isolated nuclear and cytoplasmic fractions. H3 and β-actin were used as a control protein for nucleus and cytoplasm respectively, according to a previous study (32). We observed that inhibition of CXCR4 significantly suppressed CXCL12-induced increase in cytoplasmic retention of β-catenin (Fig. 5E) and the levels of p-β-catenin\textsubscript{T120} (Fig. 5F).
Moreover, inhibition of CXCR4 also suppressed CXCL12-induced reduction in the mRNA and protein levels of TCF21 and ABCA1 (Fig. 5G-H). Together, these findings indicate that binding of CXCL12 to CXCR4 is required for the activation of the GSK3β/β-catenin{T120}/TCF21 pathway, leading to the downregulation of ABCA1 expression.

3.5 CXCL12 promotes atherosclerosis in Apoe-/- mice.

To determine the role of CXCL12 in atherosclerosis, we analyzed atherosclerotic lesions in en face aorta and aortic sinus of Apoe-/- mice. We first measured plasma levels of CXCL12 in Apoe-/- mice and found that CXCL12 was significantly increased in Apoe-/- mice transduced with LV-CXCL12 (Fig. 6A). As shown in Fig. 6B and 6C, CXCL12 profoundly increased the size of en face and aortic sinus lesions. The section staining also revealed that lipid positive area in the aortic root was remarkably increased in CXCL12-overexpressing mice, while the collagen content in lesion area of LV-CXCL12 mice did not show any statistically difference compared to the control and null groups (Fig. 6D). Since CXCL12 is a potent stimulator of cell chemotaxis, we analyzed its effect on macrophage infiltration within atherosclerotic plaques using immunohistochemistry and immunofluorescence. As expected, CXCL12 markedly increased the numbers of macrophages in atherosclerotic plaques compared to the control and null groups (Fig. 6E-F). To assess the effect of CXCL12 on lipid metabolism, we examined plasma lipid profile of Apoe-/- mice and found that HDL-C was reduced in the overexpressing CXCL12 group, while there was no significant difference in LDL-C, TG, and total cholesterol (TC) (Table 2). Collectively, our data indicate that CXCL12 lowers plasma HDL levels and promotes the formation of atherosclerotic plaques in Apoe-/- mice.

3.6 CXCL12 downregulates ABCA1 expression and reduces RCT in Apoe-/- mice.

Next, we analyzed the levels of ABCA1 in aorta and MPMs of Apoe-/- mice and found an inhibitory effect of CXCL12 on ABCA1 expression (Fig. 7A-B). Results from immunofluorescence staining further revealed that the positive areas of ABCA1 were reduced in the aortic sinus of Apoe-/- mice overexpressing CXCL12 (Fig. 7C). Consistently, we found that CXCL12 reduced cholesterol efflux from MPMs to apoA-I (Fig. 7D) and exerted an inhibitory
effect on the efficiency of RCT to plasma, the liver and feces in Apoe-/− mice (Fig. 8A-C). These
data suggest that CXCL12 downregulates ABCA1 expression and then inhibits RCT, enhancing
the development of atherosclerosis.
4. Discussion

We found for the first time that CXCL12 reduced expression of ABCA1 and subsequent cholesterol efflux from macrophages to apoA-I. Mechanistically, CXCL12 bound CXCR4 and downregulated the expression of TCF21, thereby decreasing the promoter activity of ABCA1. In addition, our findings revealed that activation of the GSK3β/β-catenin pathway served as the upstream signal for the CXCL12-induced reduction in TCF21 expression and verified that CXCL12 promoted the development of atherosclerotic lesion in Apoe-/- mice.

Increasing evidence suggests that CXCL12 involves various cardiovascular disease including atherosclerosis(10, 13, 33, 34). Most previous studies in animal models were focused on revealing the pro-atherogenic role of CXCL12 via examining the sizes, counts, and stability of atherosclerotic plaques(12). They rarely defined the underlying mechanisms of CXCL12 in vitro. Dyslipidemia is the leading cause of atherosclerosis. Chatterjee et al reported that CXCL12 induced differentiation of macrophages into foam cells, therefore proposed that CXCL12 might regulate lipid metabolism in macrophages (18). For the first time, we showed that CXCL12 reduced ABCA1 expression and subsequent cholesterol efflux from macrophages to apoA-I. ABCA1 mainly mediates the efflux of intracellular cholesterol onto lipid-free apoA-I, while ABCG1 mainly facilitates cholesterol efflux onto HDL (35-38). Therefore, CXCL12-induced decrease in cholesterol efflux to apoA-I but not to HDL implies that CXCL12 reduces cholesterol efflux by downregulating ABCA1 expression. The retention of TC in THP-1-derived macrophages also provided evidence to support CXCL12-induced cellular lipid accumulation. CXCR4, the receptor of CXCL12, is abundantly expressed in atherosclerotic plaques and acts as an important mediator of macrophage migration towards the regions of atherosclerotic lesions (14, 39). We expounded a novel mechanism of CXCR4-accelerated atherosclerosis, impairing ABCA1-mediated cholesterol efflux from macrophages via binding to CXCL12. Consistently, overexpression of CXCL12 in Apoe-/- mice led to a significant reduction in plasma HDL-C levels, which is attributed to downregulation of ABCA1 expression. Inhibition of ABCA1 expression and cholesterol efflux from MPMs in Apoe-/- mice were one of the primary reasons for the reduction in plasma HDL-C levels. HDL plays a crucial role in RCT that promotes the transport of excess cholesterol from peripheral cells to the liver for excretion(40). To evaluate the effect of CXCL12 on lipid metabolism, we measured RCT efficiency in Apoe-/- mice and found that overexpression...
of CXCL12 significantly reduced RCT. Together, our data suggest that CXCL12 plays an adverse
effect on lipid metabolism by impairing cholesterol efflux, therefore promoting atherosclerosis.

Dörings et.al recently reported that EC-derived CXCL12 serves as a contributor to plasma
CXCL12 levels and an exciter for atherogenesis(41). Consistently, our data indicate that
overexpression of CXCL12 in Apoe-/- mice enhanced the development of atherosclerosis. We
also found that incubation of macrophages with CXCL12 led to a reduction in cholesterol efflux
and a promotion of intracellular lipid accumulation. However, whether specific deletion of
CXCL12 in macrophages affects plasma CXCL12 levels and the occurrence and progression of
atherosclerosis needs to be investigated in our future research. It has been reported that treatment
of CXCL12 antibody has no effect on neointimal macrophage content in Apoe-/- mice.

Cell-specific deletion of CXCL12 in ECs or SMCs also has no significant effect on macrophage
content in atherosclerotic plaques(41, 42). However, our study revealed that global overexpression
of CXCL12 significantly increased macrophage infiltration within atherosclerotic plaques of
Apoe-/- mice. This may indicate that macrophage-derived CXCL12 could serve as an underlying
driver for macrophage infiltration. Previous studies showed that neutralization of CXCL12 in
Apoe-/- mice led to a reduction in neointimal lesion area and SMC content, suggesting the
implication of CXCL12 in the phenotype switching of SMCs(42). Given the fact that SMC
phenotypes are involved in the regulation of atherosclerotic plaque stability and atherosclerosis
progression (43), the effect of CXCL12 on SMC phenotypes will be explored in our future studies.

TCF21 has been implicated in CAD and hypertension in different populations worldwide
(44-46). Nurnberg et.al reported that TCF21 was involved in the progression of atherosclerotic
lesions(47). Conversely, Lyer et.al identified TCF21 as an atheroprotective gene(29, 48). However,
little is known about the underlying mechanism by which TCF21 regulates atherogenesis. In this
study, our findings suggest that TCF21 exerts an anti-atherogenic action by increasing ABCA1
expression, this may uncover a novel role of TCF21 in cardiovascular disease. In addition, we
found that TCF21 increased ABCA1 promoter activity, but CXCL12 elicited an inverse action.
This suggests an underlying association of CXCL12 with TCF21. Further studied showed that
CXCL12 inhibited binding of TCF21 to ABCA1 promoter region by downregulating TCF21
expression. In addition, inhibition of CXCR4 blocks the effect of CXCL12 on the expression of
TCF21 and ABCA1. Therefore, the effect of CXCL12/CXCR4 axis on ABCA1-mediated
cholesterol efflux may depend on TCF21.

β-catenin, a member of the Wnt signaling pathway, is translocated into the nucleus followed by the activation of TCF family members(49). The canonical Wnt/β-catenin pathway is triggered by hyperlipidaemia as a protective mechanism(50). Chen et al reported that β-catenin affected the uptake of lipids by macrophages (51, 52). We found for the first time that the CXCL12/CXCR4 axis has an inhibitory effect on β-catenin translocation, leading to the downregulation of TCF21 and ABCA1 expression in THP-1-derived macrophages. GSK3β elicits a common inhibitory effect on the Wnt/β-catenin pathway and is closely related to atherosclerosis. McAlpine et al found that GSK3β expedites atherosclerotic lesion and foam cell formation in Apo e-/- mice(53). Treatment of a GSK3β inhibitor can alleviate intracellular lipid accumulation(54). In consistence, we found that GSK3β impaired ABCA1-mediated cholesterol efflux and then promoted atherosclerosis, which depends on β-catenin. It has been reported that GSK3β-dependent phosphorylation of β-catenin regulates distribution of β-catenin between nucleus and cytoplasm (23-26). Here, we further explored the phosphorylation sites of β-catenin and found that phosphorylation at Thr120 in β-catenin by GSK3β was essential for CXCL12-regulated TCF21 and ABCA1 expression. Moreover, Du et al reported that cytoplasmic β-catenin retention was influenced by phosphorylation at Thr120 (55), consistent with our finding that CXCL12 increased cytoplasmic β-catenin accumulation.

Taken together, our findings reveal for the first time that CXCL12 accelerates atherosclerosis via downregulating ABCA1 expression and then reducing cholesterol efflux from macrophages. In this process, binding of CXCL12 to CXCR4 is required for the activation of the GSK3β/β-cateninT120 signaling pathway and downregulation of TCF21 expression (Fig. 8D). Therefore, CXCL12 might be a promising target to protect against atherosclerosis.

Conflicts of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.
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**Figure Legends**

**Fig.1** CXCL12 reduced ABCA1 expression for inhibition of cholesterol efflux in THP-1-derived macrophages.

The effect of CXCL12 on ABCA1 expression and cholesterol efflux. THP-1 derived macrophages were incubation with CXCL12 (80 ng/ml). (A) Analysis of mRNA and protein levels of ABCA1 using qRT-PCR and western blot, respectively. (B) Analysis of ABCA1 by immunofluorescent staining. Scale bar = 20 μm. (C) Analysis of cholesterol efflux to apoA-I and HDL using liquid scintillation counting assays. All data were shown as mean ± SD from 3 independent experiment with each performed in triplicate. *P < 0.05 vs. Control group

**Fig.2** CXCL12 downregulated TCF21 expression and inhibited binding of TCF21 to ABCA1 promoter.

The role of TCF21 in CXCL12-induced reduction ABCA1 expression. (A-B) HEK293T cells were transfected with TCF21 and ABCA1 promoter 3000, or incubation with CXCL12 (80 ng/ml) and transfected with ABCA1 promoter 3000. ABCA1 promoter activity was analyzed using the luciferase reporter gene assay. (C) THP-1-derived macrophages were transfected with ABCA1 promoter 3000 and TCF21, and then incubation with or without CXCL12 (80 ng/ml). Analysis of the action of CXCL12 on binding of TCF21 to ABCA1 promoter region using chromatin immunoprecipitation assay. (D-E) THP-1-derived macrophages were transfected with or without TCF21 and then incubation with CXCL12 (80 ng/ml). mRNA and protein levels of TCF21 (D) or ABCA1 (E) were measured using qRT-PCR and western blot, respectively. All data were shown as mean ± SD from 3 independent experiment with each performed in triplicate. *P < 0.05 vs. Control group or IgG group. *P < 0.05 vs. ABCA1 pro3000 group or TCF21 of Control group or TCF21 group.

**Fig.3** CXCL12-inhibited translocation of β-catenin was responsible for downregulation of ABCA1.

The role of β-catenin in CXCL12-reduced ABCA1 expression. THP-1-derived macrophages were incubated with CXCL12 (80 ng/ml) or/and SKL2001. (A) Analysis of protein levels of β-catenin
in nucleus and cytoplasmic using western blot. (B-C) The measurement of mRNA and protein levels of TCF21 (B) or ABCA1 (C) using qRT-PCR and western blot, respectively. (D) Analysis the levels of phosphorylated β-catenin at Thr120 and Tyr654 using western blot. (E-F) THP-1-derived macrophages were incubation with CXCL12 (80 ng/ml) or/and transfected with β-catenin mutant T120I. The measurement of mRNA and protein levels of TCF21 (E) or ABCA1 (F) using qRT-PCR and western blot, respectively. *P < 0.05 vs. Control group. #P < 0.05 vs. SKL2001 group or T120I group.

Fig. 4 GSK3β was essential for the phosphorylation of β-catenin induced by CXCL12.

THP-1-derived macrophages were incubation with CXCL12 (80 ng/ml) or/and TWS119. (A) Analysis of phosphorylation levels of GSK3β using Western blot. (B) Measurement of nuclear and cytoplasmic β-catenin using Western blot. (C) Detection of the levels of phosphorylated β-cateninY654 and β-cateninT120 using Western blot. (D-E) The measurement of mRNA and protein levels of TCF21 (D) or ABCA1 (E) using qRT-PCR and western blot, respectively. All data were shown as mean ± SD from 3 independent experiment with each performed in triplicate. *P < 0.05 vs. Control group. #P < 0.05 vs. TWS119 group.

Fig. 5 Binding of CXCL12 to CXCR4 was required for reduction in ABCA1.

The role of CXCR4 in CXCL12-induced reduction in ABCA1 expression. (A) THP-1-derived macrophages were incubation with CXCL12 (80 ng/ml). The interaction between CXCL12 and CXCR4 was examined using co-immunoprecipitation. (B-C) Detection of silent efficiency of CXCR4 siRNA using western blot and immunofluorescence assay. Scale bar = 20 μm. (D-E) THP-1-derived macrophages were incubation with CXCL12 (80 ng/ml) or/and treated with CXCR4 siRNA or inhibitor LY2510924. Analysis of the levels of phosphorylated GSKβ (D) and protein levels of β-catenin (F), and levels of phosphorylated β-cateninY654 and β-cateninT120 (E) using Western blot. (G-H) The measurement of mRNA and protein levels of TCF21 (F) and ABCA1 (G) using qRT-PCR and Western blot, respectively. All data were shown as mean ± SD from 3 independent experiment with each performed in triplicate. *P < 0.05 vs. Control group.
Fig.6 CXCL12 promoted atherosclerotic plaque formation in Apoe-/- mice.

The role of CXCL12 in the atherogenesis. Male Apoe-/- mice were treated with saline alone (Control), lentivirus or lentivirus-CXCL12 via tail vein injection and then fed the Western diet for 12 weeks. (A) Analysis of the transduce efficiency of CXCL12 using ELISA; (B) The lesion (yellow arrow) in aortic arches of Apoe-/- mice that were viewed by a stereoscopic microscope; (C) Atherosclerotic lesion areas. The entire aortas were measured by Oil Red O staining and quantified by analyzing the positive staining regions using Image J software; (D) The section of the aorta was stained with Oil Red O, HE, and Masson staining. The percent of lesion areas detected by Oil Red O staining and collagen content examined by Masson staining were quantified by analyzing the positive staining regions of aortic root sections with Oil Red O and Masson staining respectively, using Image J software. Scale bar = 300 μm. (E-F) Immunofluorescence and Immunohistochemistry analysis of CD68-postive macrophages in the section of aorta. Scale bar = 100 μm. All data were shown as mean ± SD and were collected from 7 mice every group. *P < 0.05 vs. Control group.

Fig.7 CXCL12-reduced ABCA1 expression and cholesterol efflux in Apoe-/- mice.

Male Apoe-/- mice were treated with saline alone (Control), lentivirus or lentivirus-CXCL12 via tail vein injection and then fed the Western diet for 12 weeks. (A-B) Analysis of mRNA and protein levels of ABCA1 in aorta (A) and mouse peritoneal macrophages (MPMs) (B) isolated from Apoe-/- mice using qRT-PCR and western blot, respectively. (C) The evaluation of ABCA1-mediated cholesterol efflux to apoA-I and HDL using liquid scintillation counting assays. (D) Immunofluorescence analysis of the levels of ABCA1 in the lesion of Apoe-/- mouse aorta. Scale bar = 100 μm. All data were shown as mean ± SD and were collected from 7 mice every group. *P < 0.05 vs. Control group.

Fig.8 CXCL12 inhibited RCT efficiency.

[3H]-cholesterol and ox-LDL-loading J774 macrophages were injected into the abdominal cavity of Apoe-/- mice transfected with lentivirus or lentivirus-CXCL12 and fed the Western diet. (A-C) The examination of the concentration of [3H]-tracer in serum (A), liver (B) and feces (C) using...
radioactive counting. All data were shown as mean ± SD (n=7 mice per group). *P < 0.05 vs. the Control group.

(D) A proposed model for CXCL12-regulated cholesterol efflux in macrophages. CXCL12 promotes phosphorylation of GSK3β and reduces nuclear β-catenin content. This downregulates expression of TCF21 and ABCA1, suppressing macrophage cholesterol efflux. Thus, CXCL12 exerts a stimulating effect on atherosclerosis.
Fig. 2
Fig. 3

A

B

C

D
Fig. 5

A

B

C

D

E

F

G

H
Fig. 6

A

B

C

D

E

F
Fig. 7

A) Relative ABCA1 mRNA levels in aorta tissues with ABCC1 expression by qPCR analysis. B) Relative ABCA1 protein levels in MPMs and aorta tissues by western blot analysis. C) Representative images of ABCA1 and CD31 expression in aorta tissues by immunofluorescence. D) Intracellular cholesterol efflux to apoA-I (%) and HDL (%) by MTT assay in MPMs and aorta tissues with ABCC1 expression.
Fig. 8
Table 1
The effect of CXCL12 on the lipid contents of THP-1-derived macrophages. Analysis of the levels of cellular total cholesterol (TC), free cholesterol (FC) and cholesterol ester (CE) using high-performance liquid chromatography. All data are shown as mean ± SD from 3 independent experiments with each performed in triplicate. *P < 0.05 vs. Control group.

|                      | TC (mg/g)       | CE (mg/g)      | FC (mg/g)      |
|----------------------|----------------|----------------|----------------|
| Control              | 489.73 ± 28.47 | 312.63 ± 18.69 | 177.10 ± 18.67 |
| PBS                  | 492.56 ± 30.35 | 308.28 ± 29.56 | 184.28 ± 22.49 |
| CXCL12               | 597.48 ± 23.46*| 359.32 ± 26.37*| 238.16 ± 23.41*|

Table 2
The role of CXCL12 in the regulation of plasma lipid levels in Western diet-fed ApoE-/- mice. The assessment of total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) in ApoE-/- mice by enzymatic methods. All data are shown as mean ± SD from 3 independent experiments with each performed in triplicate. *P < 0.05 vs. Control group.

|                      | TC (mmol/L)  | TG (mmol/L)  | LDL-C (mmol/L) | HDL-C (mmol/L) |
|----------------------|--------------|--------------|----------------|----------------|
| Control              | 27.18 ± 3.81 | 1.43 ± 0.35  | 25.62 ± 2.47   | 1.86 ± 0.76    |
| Null                 | 29.68 ± 4.23 | 1.56 ± 0.47  | 27.36 ± 2.21   | 1.73 ± 0.37    |
| CXCL12               | 30.28 ± 3.01 | 1.63 ± 0.56  | 28.59 ± 1.38   | 1.68 ± 0.28*   |