Intestinal epithelial chemokine (C-C motif) ligand 7 overexpression protects against high fat diet-induced obesity and hepatic steatosis in mice

Zhi-Hong Luo, Meng-Wei Niu, Shen-Hai Gong, Guang-Yan Wu, Teng Wang, Fang-Zhao Wang, Guo-Quan Wei, Zhan-Ke He, Yong Jiang, Peng Chen

Department of Pathophysiology, Guangdong Provincial Key Laboratory of Proteomics, School of Basic Medical Sciences, Southern Medical University, Guangzhou, Guangdong 510515 China.

Abstract
Background: We previously found that the intestinal epithelial chemokine (C-C motif) ligand 7 (CCL7) plays an important role in the development of toxin-induced acute liver damage. The detailed effects of intestinal epithelial CCL7 on chronic diseases; however, are still unclear. Here, we aimed to investigate the impact of intestinal epithelial CCL7 overexpression on high-fat diet (HFD)-induced obesity and steatohepatitis in mice.

Methods: Intestinal epithelial CCL7 overexpression (CCL7tgIEC) mice and their wild-type (WT) littermates were fed with normal chow or HFD for 16 weeks to induce obesity and non-alcoholic fatty liver disease. Body weight gain, as well as adipose tissue index were assessed. Liver injury was monitored by histological analysis and real time polymerase chain reaction. Gut microbial composition was analyzed by 16S rRNA gene sequencing.

Results: We found that the CCL7tgIEC mice on a HFD had markedly decreased weight gain (8.9 vs. 17.0 g, P < 0.05) and a lower adipose tissue index that include mesenteric fat (1.0% vs. 1.76%, P < 0.05), gonadal fat (2.1% vs. 6.1%, P < 0.05), subcutaneous fat (1.0% vs. 2.8%, P < 0.05) compared to WT animals. HFD-induced glucose intolerance and insulin resistance were also significantly improved in CCL7tgIEC mice compared to WT. Furthermore, HFD-fed CCL7tgIEC mice displayed less hepatic lipid accumulation and lower expression of inflammatory factors than WT mice. 16S rRNA gene sequencing demonstrated that CCL7 overexpression in intestinal epithelial cells improved HFD-induced gut microbial dysbiosis.

Conclusions: Our study revealed that CCL7 overexpression in the intestinal epithelium protects mice against the progression of diet-induced obesity, hepatic steatosis, and enteric dysbiosis.

Keywords: Chemokine (C-C motif) ligand 7; Gut microbiota; High-fat diet; Obesity; Steatohepatitis

Introduction
Obesity, a major global health concern, not only causes excessive deposition of fat in the body, but has also been identified as a key risk factor for non-alcoholic fatty liver disease (NAFLD), cardiovascular disease, diabetes mellitus, and cancer.[1] NAFLD includes extensive liver abnormalities ranging from simple steatosis to advanced cirrhosis.[2] It is also been associated with metabolic syndromes such as glucose intolerance and insulin resistance.[3] The intestines play a pivotal role not only in digestion and energy intake but also in contributing to the body’s inflammatory response during obesity and NAFLD progression.[4] An increasing body of work demonstrates that the intestinal immune microenvironment is a major component in the production of inflammatory factors and plays a significant role in diet-induced obesity and the development of related diseases.[5,6] For example, interleukin (IL)-22 was able to restore host mucosal defense and protected mice against high-fat diet (HFD)-induced metabolic disorders.[7] Additionally, IL-17 delayed the development of obesity and modulated glucose metabolism.[8]

The intestinal microflora has been widely reported to be associated with the development of obesity and related diseases.[9] First, diet-induced obesity disrupts gut microbial composition.[10,11] Gut microbiome dysbiosis was able to promote obesity and related NAFLD progression through multiple mechanisms.[12,13] Maintaining enteric eubiotic status is, therefore, recognized as an effective approach to combat diet-induced abnormalities. It is also worth noting that intestinally-derived inflammatory factors and anti-microbial...
molecules can influence the microbial composition in the gut.\(^{[14,15]}\)

Chemokine (C-C motif) ligand 7 (CCL7), also known as monocyte chemotactic protein 3, attracts various leukocytes, including monocytes and neutrophils, which regulate the function of key immune cells such as macrophage, T cells, natural killer cells, and dendritic cells, and plays an important role in the immune response.\(^{[16]}\) In addition, CCL7 is highly expressed in the intestinal lamina propria.\(^{[17]}\) We previously found that intestinal epithelial CCL7 modulates acute liver damage development.\(^{[18]}\) Here we aim to further investigate the effects of CCL7 overexpression on intestinal epithelial cells during chronic disease, specifically diet-induced obesity and NAFLD progression.

Methods

Ethical approval

All experimental procedures were approved by the local Animal Care and Use Committee of the Southern Medical University.

Animal model

CCL7\(^{tgIEC}\) (intestinal epithelial CCL7 overexpression, C57BL/6 [WT] background) mice were generated and purchased from GemPharmatech Co., Ltd (Nanjing, China).\(^{[18]}\) Five to 6-week-old female CCL7\(^{tgIEC}\) mice and their wild-type (WT) littermates were used in the study (normal chow [NC]-WT: n = 3–8, NC-CCL7\(^{tgIEC}\): n = 3–7, HFD-WT: n = 4–12, HFD-CCL7\(^{tgIEC}\): n = 4–7). Mice were housed in isolated cages in a 12 h light/12 h dark environment. Animals had access to water and food ad libitum. For the HFD-induced obesity model, mice were fed a HFD (D12492, 60 kcal% fat; Research Diets, NJ, USA) and control mice were fed NC (D12450J, 10 kcal% fat; Research Diets, NJ, USA) for 16 weeks. WT and CCL7\(^{tgIEC}\) mice were placed on the special diet at 5 to 6 weeks of age and the corresponding indicators were monitored throughout the course of the study.

Fecal DNA extraction

Fecal contents were collected from live mice, and were snap-frozen and stored at −80°C immediately. DNA was extracted from fecal contents as previously described.\(^{[18,19]}\) Briefly, the fecal contents were resuspended in phosphate buffered solution (PBS) (pH 7.4) containing 0.5% Tween 20 and subjected to three freeze-thaw cycles (−80°C/60°C) to disrupt bacterial cell membranes. The samples were then vortexed gently and DNA was extracted using the phenol-chloroform method.

16S rRNA gene sequencing, real-time polymerase chain reaction (PCR) and data analysis

The highly conserved variable region 4 (V4) of the 16S rRNA gene was amplified by PCR using 16S rRNA primers: V4F, 5’-GTGTYACAGCMGCCGCGGTAA-3’; V4R, 5’-CCGGACTACNVGGGTWTCTAAT-3’; and further sequenced by the Illumina HiSeq PE250 platform (Illumina, Inc., CA, USA). Raw sequence reads were first quality controlled using Quantitative Insights Into Microbial Ecology (QIIME) 1.9.1 (http://qiime.sourceforge.net/). Fecal microbial diversity was then assessed using LefSe (linear discriminant analysis [LDA] effect size, http://huttenhower.sph.harvard.edu/galaxy/) and QIME to determine distinct taxonomic groups. Real-time PCR was performed with an ABI 7500 (Applied Biosystems, CA, USA) sequence detection system using SYBR Green PCR master mix (Toyobo, Japan). The total reaction volume was 12 μL containing: 5 μL complementary DNA (cDNA), 0.5 μL each forward and reverse primers and 6 μL SYBR Green PCR master mix. Real-time PCR was carried out as follows: initial denaturation (2 min at 50°C; 10 min at 95°C), followed by 40 cycles of denaturation (15 s at 95°C) and annealing (1 min at 60°C). After each PCR reaction, melting curves were obtained by stepwise increases in temperature from 60 to 95°C to ensure single-product amplification. The gene expression of proinflammatory cytokines, IL-1β, IL-6, CXCL2, CXCL10, CCL4, CCL2, CCL5, CCL7, and anti-inflammatory cytokines, IL-10 and IL-13, were quantified by amplification of specific cDNA using real-time PCR. The forward and reverse primer sequences for each amplified gene were as follows: IL-1β, 5’-TGTAATGCAGCCAC- CTTTTGA-3’ and 5’-GGTTCAAGGTGTTGGAAGGAG-3’; IL-6, 5’-TGATGCACTTGGCAAAACA-3’ and 5’-ACGAGGAAATTTTCAATGGG-3’; IL-10, 5’-AGCC TATCGGAATGACGATC-3’ and 5’-GGCTCTTTGTA- AGACACCTGTTG-3’; IL-13, 5’-CTGCCCCCTCGCTTCC TCTGT-3’ and 5’-CCTGACTCTCCTGTTGAGCTTG-3’; CXCL2, 5’-GAGTTAAGGTGCTTGTG-3’ and 5’-TC CAGGTCAATGGAAGCGTC-3’; CXCL10, 5’-CTCATC TGCTTGGTCTAG-3’ and 5’-CCTATGGCCCTCA- TTCTAC-3’; CCL4, 5’-CATTAGCGTCTCTGCTG-3’ and 5’-GAACACGAGGAGGTGGGAG-3’; CCL2, 5’-CCTGCTGTTCACAGTTGTC-3’ and 5’-ATTGGGAT- CATCCTTGCTTG-3’; CCL5, 5’-GTGCCCACGTCAAG- GAGTAT-3’ and 5’-GTGGAATCTTCCGGCTGTAG-3’; CCL7, 5’-CTGCTTTTCAGCATTCAAGTG-3’ and 5’-TT CTCCTTGGGATCTTTTG-3’.

Glucose tolerance test (GTT) and insulin tolerance test (ITT)

We performed GTT and ITT on NC and HFD mice at 13 and 14 weeks, respectively. For GTT, the mice were fasted for 16 h (overnight) and 1 g/kg glucose was intraperitoneally injected. Plasma glucose levels were measured (OneTouch Ultra test strips, CA, USA) at the indicated time point (0, 30, 60, 90, and 120 min) after glucose administration. For ITT, 6 h-fasted mice were intraperitoneally injected with 0.35 U/kg insulin and plasma glucose levels were measured (OneTouch Ultra test strips) at the indicated time point (0, 30, 60, 90, and 120 min).

Hematoxylin and eosin staining (H&E)

For histopathology, the liver tissues were incubated with 10% buffered formalin, and then tissue sections were dehydrated in an ascending series of ethanol and cleared in xylene. Next, the tissues were embedded in paraaffin wax and sliced into 5-μm-thick sections. Lastly, we performed H&E staining according to the standard protocol (75°C). Sections were taken with a Zeiss microscope (Carl Zeiss Microscopy Ltd); Non-alcoholic fatty liver disease activity score (NAS) was
assessed by the methods described previously. Briefly, the parameters for steatosis, lobular inflammation, and ballooning were each graded on a scale of 0 to 3, with 0 defined as “absent” and 3 defined as “severe.”

**Oil Red O staining**
For Oil Red O staining, the liver tissues were frozen and sliced into 7-μm-thick sections and stained with fresh Oil Red O (Macklin, China). Images were taken with a Zeiss microscope (Carl Zeiss Microscopy Ltd).

**Plasma cholesterol and hepatic triglyceride measurements**
Plasma cholesterol was measured using commercially available assay kits (Jiancheng Bioengineering Institute, China) following the manufacturer’s microplate protocols. First, 2.5 μL of plasma, 2.5 μL of distilled water, and 2.5 μL of standard solution were separately added to 96-well plates, which were named as sample wells, blank wells, and standard wells, and then 250 μL of working solution was added to each well. Last, the well wall was not touched but shaken slightly when operating, and was incubated at 37°C for 10 min. The optical density value of each well was measured at a wavelength of 510 nm. The levels of plasma cholesterol were calculated.

Hepatic triglycerides were extracted with a modified chloroform-methanol Folch extraction. After liver samples (about 50 mg) with nine-fold volume PBS solution was homogenized, and centrifuged at 13,201 × g for 10 min at 4°C, the supernatant was obtained and then put into new Eppendorf (EP) tube (take 200 μL), a total of 300 μL methanol was added into the EP tube and vortexed, a total of 700 μL chloroform was added into the EP tube and vortexed, then 100 μL of distilled water was added and vortexed, and centrifuged at 13,201 × g for 10 min at 4°C, the lower layer was taken as much as possible, a total of 54 μL Triton-X/chloroform was added into the EP tube and vortexed. In the end, these EP tubes were dried in vacuo for 1.5 h. A total of 100 μL of distilled water was added into the EP tube and vortexed, then 2.5 μL of hepatic triglycerides solution was measured using commercially available assay kits (Jiancheng Bioengineering Institute) following the manufacturer’s microplate protocols.

**Statistical analysis**
All data are presented as mean ± standard deviation, and statistical analyses were performed with GraphPad Prism (version 6; GraphPad Software Inc., San Diego, CA, USA). Two-tailed Student’s t test was used for all data statistical evaluation. Statistical differences were analyzed using a significance level set at a value of P < 0.05.

**RESULTS**

**Intestinal epithelial CCL7 overexpression reduced body weight gain and fat mass in HFD-fed mice**
To assess the effect of CCL7 overexpression in the gut epithelium on HFD-induced obesity, 5- to 6-week-old WT and CCL7**tgIEC** mice were fed HFD (60% kcal from fat) or NC (10% kcal from fat) for 16 weeks [Figure 1A]. The CCL7**tgIEC** mice on HFD feeding exhibited significantly reduced body weight gain compared to HFD-fed WT mice over the course of treatment, and food intake in CCL7**tgIEC** mice was not lower than WT [Figure 1B–E]. Furthermore, the percentages of mesenteric fat (Mes), subcutaneous fat (Sub), and gonadal fat (Gon) were markedly enhanced in WT mice fed an HFD diet compared to NC, while the HFD-fed CCL7**tgIEC** group exhibited significantly lower body fat percentages than the HFD-fed WT mice [Figure 1F]. Altogether, our data suggest that CCL7**tgIEC** mice displayed a remarkable resistance to HFD-induced obesity.

**Overexpressed CCL7 in intestinal epithelial cells impedes HFD-induced glucose intolerance and insulin resistance**
Next, we examined whether intestinal epithelial CCL7 overexpression ameliorated host glucose metabolism. Primarily, intraperitoneal GTT was used to assess glucose intolerance in mice. Our data demonstrate that plasma glucose levels were markedly reduced in CCL7**tgIEC** mice compared to WT at all of the time points tested. In addition, HFD-fed CCL7**tgIEC** mice had lower glucose area under the receiver operating characteristic curve (AUC) values than their WT counterparts. Insulin tolerance, measured by intraperitoneal ITT, also showed that plasma glucose levels and AUC values were lower in CCL7**tgIEC** mice than WT up to 120 min post-injection [Figure 2A–D]. Taken together, our studies reveal that overexpression of CCL7 in intestinal epithelial cells protects mice from HFD-induced glucose intolerance and insulin resistance.

**Intestinal epithelial CCL7 overexpression improved HFD-induced fatty liver disease progress**
We further investigated whether intestinal epithelial CCL7 overexpression could ameliorate the progression of steatohepatitis. We found that the concentrations of liver triglycerides and plasma cholesterol were increased in HFD-fed mice compared with NC-fed WT mice. These values were significantly decreased in HFD-fed CCL7**tgIEC** mice compared to the HFD-fed WT group [Figure 3A and 3B]. These findings were further validated with histological analyses of liver, including H&E and Oil Red O staining [Figure 3C]. Although we did not find evidence of fibrosis in our model (data not shown), we evaluated the NAFLD progression for each group by employing NAS which includes steatosis, inflammation, and blooming. Figure 3D showed that HFD-fed CCL7**tgIEC** mice exhibited decreased NAS than HFD-fed WT animals. Moreover, the expression of hepatic pro-inflammatory factors such as CCL4, CCL5, CCL2, IL-1β, IL-6, CXCL2, CCL7, and CXCL10, were all increased in HFD-fed WT mice compared to the NC-fed WT mice and decreased in HFD-fed CCL7**tgIEC** mice compared to the HFD-fed WT mice. Besides, the expression of hepatic anti-inflammatory factors such as IL-10 and IL-13 were both decreased in HFD-fed WT mice compared to the NC-fed WT mice and increased in HFD-fed CCL7**tgIEC** mice compared to the HFD-fed WT mice [Figure 3E]. Taken together, these results indicated that CCL7 overexpression in intestinal epithelial cells reduces the progression of HFD-induced fatty liver disease.
CCL7 overexpression in the gut epithelium improved intestinal dysbiosis induced by HFD

Intestinal epithelial cells produce a variety of immune products, including anti-microbial molecules, which have the potential to alter the host’s intestinal microbiota. CCL7, one of many chemotactic factors produced, participates in the inflammatory response through binding to its receptor to regulate the recruitment of immune cells. However, few studies have examined the effects of CCL7 overexpression on the intestinal microbiota. We compared the composition of gut microbiota of CCL7tgIEC mice and their WT littermates by 16S rRNA gene sequencing in both the NC- and HFD-fed groups. The 16S rRNA datasets show that in the NC group, the microbiota of CCL7tgIEC mice is not clustered separately from that of WT littermates based on the principal component analysis. However, in the HFD group, the microbiota of CCL7tgIEC mice are clustered separately from their WT littermates [Figure 4A]. The LDA scores show that the relative abundance of the Lactococcus was enriched in HFD-fed CCL7tgIEC mice [Figure 4B]. At the phylum level, the Proteobacteria were significantly increased in HFD-fed WT mice compared to the NC-fed WT group. Additionally, within the HFD group, the CCL7tgIEC mice showed a decreased trend in the abundance of Proteobacteria than WT [Figure 5A and 5B]. At the family level, the relative abundance of Desulfovibrionaceae was significantly increased in WT mice fed an HFD diet compared to an NC diet, while HFD-fed CCL7tgIEC mice also showed a decreased trend in the abundance of

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**Figure 1:** Intestinal epithelial overexpression of CCL7 protects against obesity in mice. (A) Schematic of WT and CCL7tgIEC mice fed NC or HFD. (B) Representative images of mice after 16-weeks on an HFD diet. (C) Body weight gain (*Comparison between HFD-WT and HFD-CCL7tgIEC*). (D) Quantitative figure of weight gain at 16-week. (E) Food intake of the mice. (F) Index of mesenteric fat, gonadal fat, and subcutaneous fat. NC-WT: n = 4–8, NC-CCL7tgIEC: n = 5–7, HFD-WT: n = 11–12, HFD-CCL7tgIEC: n = 7. Data represent mean ± standard deviation. *P < 0.05. BW: Body weight; CCL7: Chemokine (C-C motif) ligand 7; Gon: Gonadal; HFD: High-fat diet; Mes: Mesenteric; NC: Normal chow; Sub: Subcutaneous; WT: Wild-type.
Desulfovibrionaceae than their WT counterparts [Figure 5C and 5D]. Thus, our data indicated that HFD-induced gut microbial changes could be reversed by CCL7 overexpression in intestinal epithelial cells.

Discussion

We previously found that CCL7tgIEC mice exhibited a mild increase in inflammation of the liver as evidenced by increased cytokines and chemokines gene expression compared to the control group. Our results presented in this paper confirm this observation [Figure 3]. Given the recently published data that mild inflammation is a driving factor for obesity, we hypothesized that CCL7tgIEC mice would develop severe NAFLD and be more prone to obesity after being subjected to a HFD. Unexpectedly, the CCL7tgIEC mice showed less body weight gain and improved NAFLD syndrome than their WT littermates. The mechanism underlying this phenotype is complex; however, we speculate that the improvement in enteric dysbiosis seen in the gut of CCL7tgIEC mice on an HFD may play a more prominent role in disease progression than mild inflammation.

Previous studies have reported that obesity and metabolic syndromes are associated with variations in the intestinal microbiota. Interestingly, the metabolic syndromes and differences in the gut microbiota have been well discussed, which is characterized by the proliferation of potentially harmful bacteria and suppression probiotics. It is also reported that microbiota structure or derived metabolites had significant differences in chronic colitis status compared to control group. However, the interactive relationship between obesity, the gut microbiome, and intestinal immunity is still unclear. In our study, based on 16S rRNA gene sequencing analysis of mouse fecal microbiota, we found that CCL7 overexpression in intestinal epithelial cells caused alterations in the intestinal microbial landscape upon HFD challenge, which may, in turn, interfere with high-fat-diet-induced obesity, insulin resistance, and the development of non-alcohol fatty liver disease. In recent years, some studies reported that intestinal microbial dysbiosis is an important factor in obesity. Probiotics and prebiotics have been reported to be an important treatment to combat NAFLD and obesity. Specifically, Lactobacillus spp., Akkermansia muciniphila, and Bifidobacterium spp have been identified as beneficial species as these strains improved obesity and insulin resistance in rodents. Most notably, the abundance of bifidobacteria, a group of bacteria that has been shown to protect the intestinal barrier of animals that consume a HFD, and the sulfate-reducing Desulfovibrionaceae family are known to...
Figure 3: Intestinal epithelial overexpression of CCL7 ameliorates HFD-induced steatohepatitis in mice. (A) Hepatic triglyceride levels. (B) Cholesterol levels in plasma. (C) Oil-red O (upper panel) and H&E (lower panel) staining of the liver. Original magnification, ×200. (D) NAS of H&E staining. (E) mRNA levels of hepatic pro-inflammatory and anti-inflammatory factors. NC-WT: n = 3–7, NC-CCL7tgIEC: n = 3–5, HFD-WT: n = 4–11, HFD-CCL7tgIEC: n = 4–7. Data represent mean ± standard deviation. P < 0.05. CCL7: Chemokine (C-C motif) ligand 7; CHOL: Cholesterol; HFD: High-fat diet; NAS: Non-alcoholic fatty liver disease activity score; NC: Normal chow; TG: Triglyceride; WT: Wild-type.
be enhanced in animals suffering from glucose intolerance.\cite{37} Our findings demonstrate that the relative abundance of Desulfovibrionaceae showed a decreased trend in the HFD-fed CCL7\textsuperscript{tgIEC} mice compared to WT. Therefore, the potential effect of intestinal epithelial overexpression of CCL7 on these bacteria may contribute to the improvement of obesity. The mechanism by which CCL7 modulates enteric microbiota composition may be the result of the altered immune microenvironment that occurs in the presence of CCL7 overproduction in the intestine. However, the details of this mechanism require further investigation.

Several limitations need to be addressed in the current study. First, the specific mechanism that causes CCL7 overexpression in the intestinal epithelium to impact the composition and function of the gut microbiota remains unclear. Second, there is currently no information on the effects of the loss of intestinal epithelial CCL7 on high-fat-diet-induced obesity, insulin resistance, and NAFLD.
Figure 5: CCL7 overexpression influenced the composition of fecal microbiota in mice on an HFD diet. (A) Relative abundance at the phylum level of all the four groups. (B) Relative abundance of Proteobacteria. (C) Relative abundance at the family level of all the four groups. (D) Relative abundance of Desulfovibrionaceae at the family level. NC-WT: \( n = 8 \), NC-CCL7tgIEC: \( n = 5 \), HFD-WT: \( n = 7 \), HFD-CCL7tgIEC: \( n = 5 \). Data represent mean ± standard deviation. * \( P < 0.05 \). CCL7: Chemokine (C-C motif) ligand 7; HFD: High-fat diet; NC: Normal chow; WT: Wild-type.
In conclusion, our study demonstrates that overexpression of CCL7 in the intestinal epithelium ameliorates HFD-induced obesity, insulin tolerance, and the development of NAFLD. These results may be due to the reversal of HFD-induced alterations to the host’s intestinal microbial community.

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Conflicts of interest

None.

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