

Original Paper

Elevated Interleukin-38 Level Associates with Clinical Response to Atorvastatin in Patients with Hyperlipidemia

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Key Words

Hyperlipidemia • IL-38 • Interleukin-38 • Atorvastatin • Atherosclerosis

Abstract

Background/Aims: Hyperlipidemia is a risk factor for various cardiovascular and metabolic disorders. And it is tightly related to chronic inflammation. Interleukin-38 (IL-38) represents a new member of anti-inflammatory cytokines. Thus we studied the important role of IL-38 in hyperlipidemia development and treatment. **Methods:** The mRNA level of IL-38 in PBMCs (peripheral blood mononuclear cells) and serum IL-38 levels in hyperlipidemia patients and healthy controls were measured by real-time polymerase chain reaction (RT-PCR) and enzyme-linked immunoassay (ELISA). The hyperlipidemia patients were further divided into two groups (Sensitive and Resistant Group) according to their clinical response to Atorvastatin therapy. Finally, the effects of IL-38 on hyperlipidemia was evaluated in the mice model. **Results:** Data showed that the IL-38 mRNA and serum protein levels were higher in patients with hyperlipidemia compared with healthy controls. And the IL-38 mRNA and serum protein levels were higher in patients sensitive to Atorvastatin therapy than the resistant group. *In vitro*, IL-38 inhibited the production of IL-6, IL-1 β and CRP in PBMCs of patients with hyperlipidemia. In the mice model of hyperlipidemia, IL-38 was also elevated during the hyperlipidemia development. Furthermore, the IL-38 over-expressed by adeno-associated virus significantly inhibited the hyperlipidemia development, inflammatory factor secretion and also the atherosclerosis process. **Conclusion:** Thus our data showed that IL-38 might present protective effects on hyperlipidemia treatment.

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Published by S. Karger AG, Basel**Introduction**

High plasma lipids are risk factors for various cardiovascular and metabolic disorders such as atherosclerosis, obesity and metabolic syndrome [1-4]. Cardiovascular disease itself is the leading cause of death in the world [5, 6]. Statins represent a highly effective therapeutic approach for reducing plasma lipids and related complications [7]. However, the

responsiveness to statin is influenced by environmental or genetic factors [8]. Only 20–40% individuals could benefit from the statin therapy, highlighting a need for new approaches to lower plasma lipids [9–15].

Chronic inflammation is tightly associated with hyperlipidemia [16–19]. Interleukin-38 (IL-38) is a new identified anti-inflammatory factor in the IL-1 ligand family [20, 21]. IL-38 binds to IL-36R similar to IL-36Ra, which can inhibit the pro-inflammation function of IL-36 [22]. Moreover, polymorphisms in IL-38 were associated with CRP concentrations in humans [23] and were found to be significantly associated with coronary artery disease (CAD) [24]. In addition, IL-38 mRNA was found in human atheromatous plaques of coronary artery disease patients [25]. These results suggest an important role for IL-38 in cardiovascular diseases, indicating that IL-38 might also play an important role in hyperlipidemia which contributes to cardiovascular disease development. Thus, we conducted the present study to investigate the role of IL-38 in hyperlipidemia development and treatment.

Materials and Methods

Patients

Patients with primary hyperlipidemia were selected from the outpatients, who were evaluated for the presence of CAD, at the cardiovascular disease clinics affiliated with Tianjin Chest Hospital between January 2010 and October 2016. The study protocol was approved by the Ethics Committees of Tianjin Chest Hospital (code number 200957E45). All the patients signed an informed consent form. Women as well as individuals with serum triglycerides above 400 mg/dl or with thyroid disease (TSH \geq 5 or \leq 0.4 μ U/dl), high cardiovascular risk, renal failure (SCr \geq 2 mg/dl for women, SCr \geq 2.5 mg/dl for men), liver diseases (ALT \geq 40 mg/dl), diabetes mellitus (who had two consecutive FBS \geq 126 mg/dl), severe psychological disorders, physical disabilities, cancer, Alzheimer's disease, AIDs (acquired immune deficiency syndrome) or other communicable diseases, and individuals treated with oral contraceptives and lipid-lowering drugs were not enrolled in the study. Four weeks before the study, all the subjects visited a dietitian and were instructed to consume a low-cholesterol diet (total daily fat intake 25 to 35% of total calories, trans fats to less than 7% of calories) [1, 26, 27]. Information on age, gender, body mass index (BMI), blood pressure or anti-hypertensive drug history, physical activity (20 minutes walking per day), family history and past medical history of each patient were recorded. Alcoholic and smoker patients were excluded from the study due to the limited numbers.

A total of 424 hyperlipidemia patients were recruited, whose TG and TC values were higher than 150 and 200 mg/dl, respectively. Healthy controls (n=408) with normolipidemia were recruited from our health examination center at the same time (TG of <150 mg/dl and TC of <200 mg/dl) [1, 26, 27]. All participants signed an informed consent form. All methods were performed in accordance with the relevant guidelines and regulations for both mice and human.

Patients were treated with 10 mg Atorvastatin orally once daily for four weeks. The patients response to the Atorvastatin were defined as their ideal LDL goal (LDL<115 mg/dl) [1, 26, 27]. The cardiovascular risk was assessed by Framingham Risk Score (FRS) and Atherosclerotic Cardiovascular Disease risk score (ASCVD) [28–30].

Lipid measurement

One week before and four weeks after Atorvastatin administration, peripheral blood samples were collected from the individuals after an overnight fasting (8–12 h) [1]. Serum samples were collected, stored at 4 °C, and analyzed within 24 hours. Total serum cholesterol, HDL, LDL and triglyceride concentrations were determined on an Abbott Architect C-16000 by commercially available colorimetric kits (Abbott Architect C-16000 dedicated kits). Before and after the administration of Atorvastatin, ALT and creatinine kinase were determined to detect the possible adverse drug reactions.

RNA extraction and real-time polymerase chain reaction (RT-PCR)

Total RNA was extracted from PBMCs with Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Then the quantity and purity of RNA was determined by absorbance on a

FilterMax F5 Multi-Mode Microplate Reader (Sunnyvale, California, USA) at 260 nm and 280 nm. Samples with ratios from 1.8 to 2.0 were accepted for next reverse transcription reaction. cDNA was prepared by using the iScript™ cDNA Synthesis kit (Bio-Rad, USA). β -actin was used as internal control. RT-PCR amplification reaction was prepared with the SYBR Green PCR kit (Bio-rad, USA) and performed using the 7500 fast Real-Time PCR system (Applied Biosystems, USA). PCR products were verified by melting curve analysis. Relative mRNA levels of target genes were calculated by the $2^{-\Delta\Delta Ct}$ method.

Enzyme-linked immunoassay (ELISA)

The levels of plasma IL-38 (Adipogen), IL-6 (BD Biosciences), IL-1 β (BD Biosciences) and CRP (Biocalvin) were measured by an enzyme-linked immunosorbent assay (ELISA), following the manufacturer's instructions. The minimal detectable concentration was 40 pg/mL for IL-38. The ELISA intra-assay and interassay coefficients of variation were <5% and <10%, respectively. All of the samples were measured in duplicate.

Recombinant human IL-38 protein expression and purification

Interleukin-38 gene (Homo Species) was amplified from cDNA of peripheral blood mononuclear cells. The PCR fragments were double digested with restriction endonucleases and ligated into the prokaryotic expression vector. The fusion protein was expressed in a stable prokaryotic expression system. The plasmids of positive clones were then sequenced by Sanger method with 100% identify with the published sequence. The induced and un-induced cultures were analyzed by SDS-PAGE to identify the expression of recombinant protein. The harvested cells were resuspended in NaCl-Tris-HCl buffer, sonicated in an ice bath, 12000 rpm centrifuged for 30 min, and then the supernatant were collected. The supernatant were added to His Trap HP, 1 ml column (GE) that had been equilibrated with NaCl-Tris-HCl buffer. Different concentrations of imidazole buffer were used to elute the recombinant protein. Collected target protein peaks were examined by SDS-PAGE electrophoresis and immunoblot analysis using anti-human IL-38 antibody (Abcam, UK). The eluted recombinant protein was dialyzed in PBS at 4°C for overnight. The concentration was detected by Bradford methods, and the recombinant protein was stored at -20°C.

Cell culture condition

Culture medium is consisted of RPMI 1640 (Hyclone, Thermo, USA) supplemented with 10% Fetal Calf Serum (Hyclone, Thermo, USA), 100 IU/ml penicillin and 100 μ g/ml streptomycin (Hyclone, Thermo, USA). Whole PBMCs were cultured in 24 well, flat-bottomed plates (5×10^5 cells/ml/well) for 24 hours. PBMCs were stimulated with or without human recombinant IL-38 at 50 ng/ml for 24 hours and then incubated further with LPS (1 μ g/ml) for 6 hours. Total RNAs were extracted, and cytokine transcriptions were analyzed by RT-PCR. Culture supernatants were harvested and froze at -80°C for later cytokine analysis by ELISA.

Animal study

The 6–8 weeks old ApoE-deficient (ApoE^{-/-}) mice on a C57Bl/6J background (The Jackson Laboratory, Bar Harbor, ME) were housed in specific pathogen-free conditions. Only male mice were used in these experiments to avoid the effects of hormonal changes on plasma lipids. The study was approved by the Research Ethics Committee of Tianjin Chest Hospital. Mice were housed in an air-conditioned room at 22°C \pm 0.5°C with a 12-hour lighting schedule (7:00–19:00). Mice were kept on a normal chow diet (Research Diets) or fed a high-cholesterol diet (HCD; 21% fat and 0.15% cholesterol; Research Diets).

Diet-induced atherosclerosis was induced by feeding ApoE^{-/-} mice an HCD for 12 weeks. Before tissue or blood collection, mice were starved for 16 hours, unless otherwise indicated. Blood was collected via the tail vein. The aorta and carotid arteries were harvested after in situ perfusion fixation with 4% paraformaldehyde (Carl Roth GmbH, Karlsruhe, Germany). For quantification of atherosclerosis, animals were euthanized by intraperitoneal pentobarbital injection (100mg/kg) and aortic trees were dissected and stained with oil red O. The *en face* lesion size was analyzed with the ImageJ software.

Production and in vivo Delivery of Adeno-associated Virus

Vector construction, production, and *in vivo* delivery of adeno-associated virus (AAV) were performed based on the AAV helper-free system (Agilent). The recombinant adenoviral vector pAAV-IL38 was

constructed by cloning the cDNA encoding region into pAAV-ITR. The vector pAAV-GFP encoding green fluorescence protein was used as a negative control. Recombinant AAVs were produced by HEK293 cells (ATCC) transfected with pAAV-ITR vectors together with pAAV-RC and pHelper plasmids, and then purified by discontinuous iodixanol gradient centrifugation. Purified recombinant AAVs were concentrated and desalted by centrifugation through Amicon Ultra 30K filters (Millipore). For *in vivo* delivery, recombinant AAVs equivalent to 1.0×10^{12} viral genome copies were delivered through mouse tail vein. AAVs were injected at week 0, 3, 6, 9 after feeding the HCD.

Statistical analysis

Data were expressed as mean \pm SE or median plus interquartile range and analyzed by Graphpad Prism V.5.00 software (GraphPad Software, San Diego CA, USA). Comparisons between groups were made using nonparametric Mann-Whitney U-test. *p* values under 0.05 were considered statistically significant.

Results

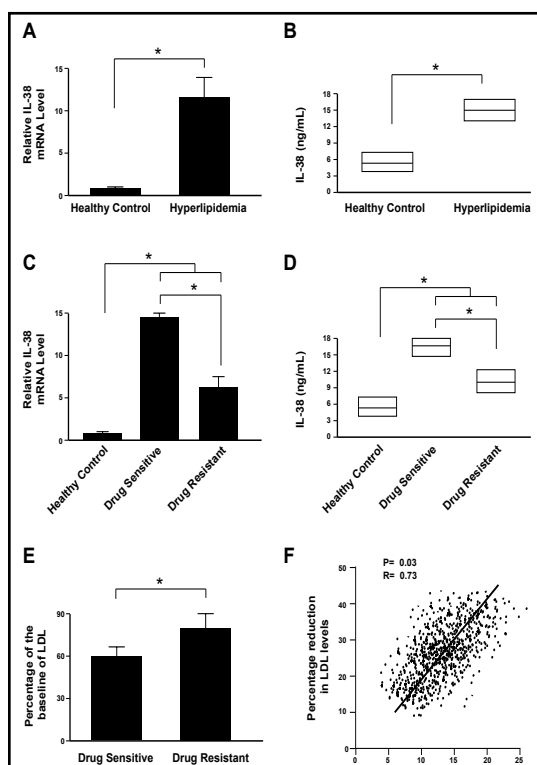
Elevated IL-38 in Patients with Hyperlipidemia

The clinical characteristics of all patients and healthy controls were summarized in Table 1. The healthy and hyperlipidemia patients did not differ in age and BMI (Table 1). Hyperlipidemia patients' TC, TG and LDL levels were significantly higher than those in control (Table 1) while the HDL levels were lower (Table 1). Furthermore, both mRNA and protein levels of IL-38 were significantly elevated in the hyperlipidemia patients than healthy controls (Table 1, Fig. 1A-1B).

IL-38 Associates with Clinical Responses to Atorvastatin

Then the patients were divided into two groups, Atorvastatin sensitive and resistant

Fig. 1. Elevation of IL-38 level in hyperlipidemia patients and association with clinical response to Atorvastatin therapy. (A) IL-38 mRNA levels in PBMCs from hyperlipidemia patients (n=424) and healthy controls (n=408) were determined by real-time PCR. * *P*<0.05. (B) IL-38 protein levels in the serum from hyperlipidemia patients (n=424) and healthy controls (n=408) were determined by ELISA. Result is depicted as box plots; middle line indicates median; bottom of box, 25th percentile; and top of box, 75th percentile. * *P*<0.05. (C) IL-38 mRNA levels in PBMCs from hyperlipidemia patients sensitive to statin treatment (n=137), resistant to statin treatment (n=287) and healthy controls (n=408) were determined by real-time PCR. * *P*<0.05. (D) IL-38 protein levels in the serum from hyperlipidemia patients sensitive to statin treatment (n=137), resistant to statin treatment (n=287) and healthy controls (n=408) were determined by ELISA. Result is depicted as box plots; middle line indicates median; bottom of box, 25th percentile; and top of box, 75th percentile. * *P*<0.05. (E) Percentage change of the LDL level comparing with the baseline values after 10 mg Atorvastatin treatment between the hyperlipidemia patients sensitive (n=137) and resistant to statin treatment (n=287). * *P*<0.05. (F) Correlation between plasma IL-38 (ng/mL) and the percentage of the reduction of LDL levels. *P*<0.05. Inset corresponds to Pearson's R correlation and corresponding *P* value.



group, according to their response to Atorvastatin therapy following four weeks of treatment with 10 mg/day Atorvastatin. The patients sensitive to the Atorvastatin (Drug Sensitive) were defined as their ideal LDL goal ($\text{LDL} < 115 \text{ mg/dl}$) [1]. Otherwise, they were defined as Atorvastatin resistant group (Drug Resistant). Data showed that both the mRNA and protein levels of IL-38 were significantly highly expressed in the patients sensitive to Atorvastatin therapy than the resistant group (Fig. 1C-1D). And the Atorvastatin was more effective in the Drug Sensitive group than the Drug Resistant group (Fig. 1E). Furthermore, the plasma IL-38 level was positively correlated with the percentage of the reduction of LDL levels (Fig. 1F). Thus the IL-38 level might associate with the clinical responses to the Atorvastatin therapy.

IL-38 Suppress Inflammatory factors expression in Hyperlipidemia Patients

As hyperlipidemia is tightly associated with inflammation [16-19] and the IL-38 represents a new identified anti-inflammatory factor [20, 21], we further studied whether IL-38 could suppress the inflammatory factors expression in the cultured hyperlipidemia patients' PBMCs treated with recombinant human IL-38 protein. As expected, the pro-inflammatory factors IL-6, CRP and IL-1 β were highly expressed in the hyperlipidemia patients' PBMCs than healthy control at both mRNA level and protein level (Fig. 2A-2B). Interestingly, administrating human recombinant IL-38 at 50 ng/ml for 24 hours could significantly suppress their expression at both mRNA level and protein level (Fig. 2A-2B).

IL-38 Suppresses Hyperlipidemia and Atherosclerosis by Limiting Inflammation

To further investigate the role of IL-38 in hyperlipidemia development and treatment, ApoE^{-/-} mice were fed with an HCD (high-cholesterol diet) for 12 weeks. The

Table 1. Clinical features of the participants. All patients and healthy controls were male. BMI: body mass index; TC: total cholesterol; TG: Triglyceride; HDL: high-density lipoprotein; LDL: low-density lipoprotein; IL-38: Interleukin-38. FRS: Framingham risk score; ASCVD: atherosclerotic cardiovascular disease risk score. *P < 0.05 indicates a significant difference. Data were shown as mean \pm SE or median (interquartile range)

	Hyperlipidemia (n=424)	Control (n=408, %)	P value
Age (years)	54.5 \pm 4.4	57.01 \pm 5.7	0.245
FRS	3.6 \pm 1.2	3.1 \pm 1.0	0.248
ASCVD	2.4 \pm 1.4	1.9 \pm 1.1	0.154
BMI (kg/m ²)	24.05 \pm 1.45	24.24 \pm 1.95	0.857
TC (mg/dl)	278.6 (256.8-311.5)	169.5 (148.6-178.3)	0.005*
TG (mg/dl)	228.9 (198.5-295.6)	95.6 (78.6-110.8)	0.001*
LDL (mg/dl)	152.5 (135.8-175.9)	88.6 (68.5-108.9)	0.007*
HDL (mg/dl)	31.9 (25.7-44.2)	45.6 (30.2-56.8)	0.044*
IL-38 (ng/mL)	15.9 \pm 3.3	5.1 \pm 1.2	<0.001*

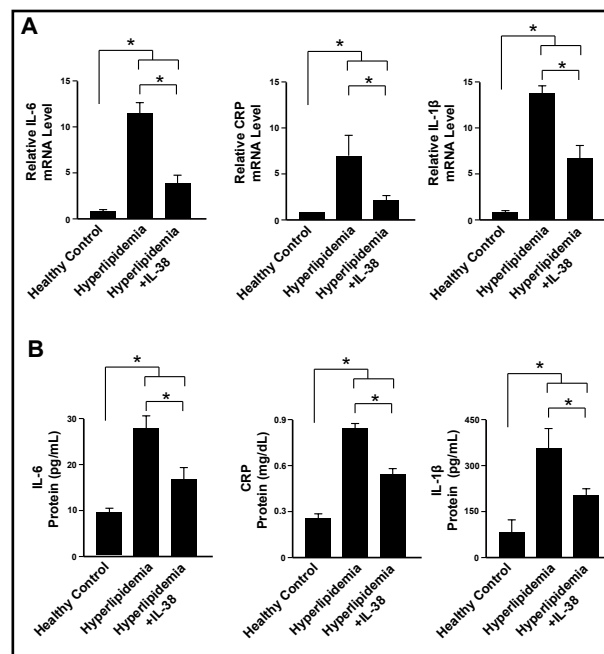
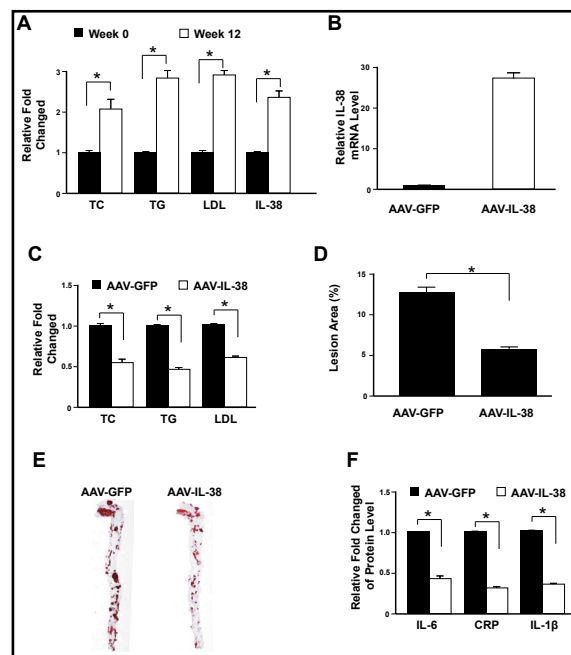


Fig. 2. Recombinant IL-38 protein suppresses the secretion of pro-inflammatory factors. PBMCs from hyperlipidemia patients (n = 424) were stimulated with or without IL-38 (50 ng/ml) for 24 hour. PBMCs from healthy controls (n = 408) were used as controls. The pro-inflammatory factor IL-6, CRP and IL-1 β were analyzed. The cells were lysed for mRNA analysis via RT-PCR (A) and the supernatants were examined using ELISA (B). * P < 0.05. CRP: C-reactive protein; IL: interleukin.

Fig. 3. IL-38 overexpression suppresses the hyperlipidemia and atherosclerosis in the mice model. (A) Hyperlipidemia mice model was established by feeding ApoE^{-/-} mice with HCD (high-cholesterol diet) for 12 weeks. The TC, TG, LDL and IL-38 levels were measured (n=12). * P<0.05. (B) IL-38 overexpression was performed by delivering AAV-IL-38 every 3 weeks. The mRNA of IL-38 in the thoracoabdominal aorta was determined by qPCR (n=12). * P<0.05. (C) The serum TC, TG and LDL levels were measured in the mice treated with AAV-GFP or AAV-IL-38 (n=12). * P<0.05. (D) The atherosclerosis lesion areas in the thoracoabdominal aorta were measured in the mice treated with AAV-GFP or AAV-IL-38 (n=12). * P<0.05. (E) Representative figures for the atherosclerosis lesion areas in the thoracoabdominal aorta. (F) The pro-inflammatory factor IL-6, CRP and IL-1 β levels in the mice treated with AAV-GFP or AAV-IL-38 were analyzed (n=12). * P<0.05. CRP: C-reactive protein; IL: interleukin; TC: total cholesterol; TG: triglyceride; LDL: low-density lipoprotein cholesterol; AAV: adeno-associated virus.



TC, TG and LDL levels were elevated at week 12 comparing with week 0 after HCD treatment (Fig. 3A). Furthermore, the IL-38 level was also increased, which is in accordance with the clinical data (Fig. 3A, Fig. 1A-1B). Overexpressing the IL-38 via AAV delivery every 3 weeks significantly decreased the TC, TG and LDL levels (Fig. 3B-3C). In addition, the size of the atherosclerotic lesions in the thoracoabdominal aorta was reduced by AAV-IL38 (Fig. 3D-3E). Inflammation is a key component during the initiation and progression of atherosclerosis [31]. Therefore, we also measured the pro-inflammatory factor IL-6, CRP and IL-1 β expression in the hyperlipidemia mice model treated with or without AAV-IL38. Data showed that overexpressing IL-38 significantly suppressed these pro-inflammatory factors' expression (Fig. 3F). These data suggest that the alleviated atherosclerotic lesion formation by IL-38 overexpression might be attributed to inflammation suppression.

Discussion

At present, hyperlipidemia is an important global health concern [5]. Hyperlipidemia is the presence of increased or abnormal levels of lipids and/or lipoproteins in the blood and a strong risk factor for atherosclerosis, stroke, CAD (coronary artery disease) and myocardial infarction (MI), such as elevated levels of total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL) or decreased level of high-density lipoprotein cholesterol (HDL) [1, 32]. Furthermore, hyperlipidemia also associates with obesity, insulin resistance and nonalcoholic fatty liver disease (NAFLD), which are important features of metabolic syndrome [33].

Because chronic inflammation is tightly associated with hyperlipidemia [16-19] and IL-38 is a new identified anti-inflammatory factor [20, 21], we studied the important role of IL-38 in hyperlipidemia development and treatment in the current study. The mRNA level of IL-38 in PBMCs and also the protein level in the serum were higher in hyperlipidemia patients compared with the healthy controls. Furthermore, the IL-38 levels were higher in patients sensitive to Atorvastatin therapy than the resistant group. *In vitro*, IL-38 inhibited the production of IL-6, IL-1 β and CRP in PBMCs of patients with hyperlipidemia.

In order to confirm the reliability of the clinical results, a high-fat diet induced hyperlipidemia mice model was used. In the mice model of hyperlipidemia, IL-38 was also elevated during the hyperlipidemia development. And the IL-38 overexpression by AAV significantly inhibited the hyperlipidemia development, inflammatory factor expression and also the atherosclerosis process. Thus our data showed that IL-38 might present protective effects on hyperlipidemia treatment.

Atherosclerosis is a multi-factorial disorder of the heart vessels that develops over decades, coupling inflammatory mechanisms and elevated total cholesterol levels under the influence of genetic and environmental factors. Without effective intervention, atherosclerosis consequently causes coronary heart disease (CHD), which leads to increased risk of sudden death [18]. It is a chronic inflammatory disorder of the vessel wall, where both innate and adaptive immune responses influence disease progression [19]. This involves: impairment of endothelial cell (EC) function; accumulation of cholesterol in subendothelial macrophage-derived foam cells; adherence and recruitment of leukocytes into the arterial wall; proliferation and migration of smooth muscle cells into the intima; activation and aggregation of platelets; T cell activation; and production of inflammatory cytokines [34]. The innate signals within the lesion can arise from various sources and promote atherosclerosis through inflammatory processes. Oxidized low-density lipoprotein (LDL) accumulation, starting in the fatty streaks, promotes the inflammatory response, which most likely continues throughout lesion development. Furthermore, pathogenic infection and endogenous danger signals increase during tissue injury and have been implicated as inducers of lesion inflammation [34].

Previous studies have shown that targeting inflammation might represent an alternative approach to suppress the hyperlipidemia and also the atherosclerosis [31, 35, 36]. Our data here demonstrated that IL-38, a newly identified anti-inflammatory factor, also showed protective effect during hyperlipidemia treatment in the clinic and alleviated atherosclerosis development in the mice model. Thus, IL-38 could be a potential candidate for immunotherapy in the hyperlipidemia and atherosclerosis. However, the manner in which IL-38 sensitizes hyperlipidemia patients to statin therapy is not well known. Therefore, further studies are needed to determine the exact mechanism of action of IL-38 in the hyperlipidemia.

Acknowledgements

This work was supported by the Fund for key project of Science and Technology of Tianjin Health and Family Planning Commission (2015KR07) and key project of Science and Technology of Tianjin Health and Family Planning Commission (13KG131).

NY design and conduct the experiment; YS, BD, YL, LK and JY conduct the experiment and collect the data; QQ design the experiment, analyze the data and write the manuscript.

Disclosure Statement

The authors declare that they have no competing interests.

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