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C5L2 Deficiency Enhances Development of Atherosclerosis in ApoE Knockout Mice

Yan Liu[1,2], Alexandre Fisette[1], Marc Lapointe[1], Katherine Cianflone[1*]

[1]Centre de Recherche de l'Institut Universitaire de Cardiologie & Pneumologie de Québec, Université Laval, Québec, Canada
[2]Department of Pediatrics, Tongji Hospital, Huazhong University of Science and Technology, Wuhan, China
Email: yan.liu.2@ulaval.ca, alexandre.fisette@criucpq.ulaval.ca, Marc.Lapointe@criucpq.ulaval.ca, [*]katherine.cianflone@fmed.ulaval.ca

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Abstract

Background: The complement system is important in development of atherosclerosis via regulation of lipid and glucose metabolism as well as inflammation. Aim: The aim of the present study was to further analyze the contribution of C5L2 to the development of atherosclerosis. We proposed that, with DIO feeding, C5L2 deficiency would promote a phenotype that encourages atherosclerosis development. Coupled to ApoE deficiency, double knockout (2KO) mice would show exacerbated atherosclerotic plaque formation. Methods: First, Wildtype (WT) and C5L2-/-(C5L2KO) and subsequently, ApoE-/-(ApoEKO) and C5L2/ApoE double knockout mice were placed on diets inducing obesity (DIO) or standard chow diet for 12 - 15 weeks. Plasma lipids, glucose, cytokines and hepatic glycogen and lipid contents, mRNA levels and enzyme activities and atherosclerotic plaque size were measured. Results: C5L2KO had increased hepatic glucose oxidation (+90%, $p < 0.001$), reduced liver glycogen content on chow diet (−34%, $p < 0.05$) but increased with DIO (+51%, $p < 0.05$) vs WT. Glucose clearance was delayed in C5L2/ApoE-2KO vs ApoEKO mice with chow ($p < 0.0001$) and DIO diet ($p = 0.0026$). C5L2KO mice had increased hepatic lipid content and fatty acid synthesis but decreased lipid oxidation vs WT. Plasma cholesterol was further elevated in C5L2/ApoE-2KO vs ApoEKO with DIO feeding ($p < 0.05$). Hepatic cytokine expression was increased in C5L2KO mice compared to WT mice. Atherosclerotic plaque size was increased in C5L2/ApoE-2KO mice compared with apoEKO on chow ($p < 0.05$) and DIO regimen ($p < 0.001$). Conclusions: C5L2 disruption worsens glucose and lipid metabolism, increases hepatic and circulating inflammation, and aggravates atherosclerosis.

Keywords

Atherosclerosis, C5L2, Inflammation, Metabolism, Lipid, Glucose

[*]Corresponding author.

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1. Introduction

Atherosclerosis is a major cause of cardiovascular morbidity and mortality in the Western world and of growing importance in developing countries. Its etiology has long been considered a process of lipid accumulation in arterial walls accompanied by smooth muscle proliferation [1]. Nowadays, atherosclerosis is also seen as an inflammatory disease driven by the interaction of inflammatory cells and the vascular wall [2]. The arterial wall thickens as a result of atheromatous plaque formation, and lipids and inflammation play a central role in the pathogenesis of these plaques [3]. Atherosclerotic lesions are well-linked with diabetes development, and this is partly mediated through the complement system (as very recently reviewed by Hess K [4]). Further studies also reveal the implication of the complement system in the development and exacerbation of various cardiometabolic diseases which include Metabolic Syndrome, dyslipidemias and cardiovascular disease as well as insulin resistance [5] [6].

The complement system is an important constituent of the immune system and a number of complement components have been shown to have dual immune and metabolic roles [7]. Although controversial, the seven-transmembrane receptor C5L2 is proposed as a functional receptor for Acylation Stimulating Protein (ASP) and is expressed in multiple tissues such as muscle, spleen, liver, brain as well as adipose tissue [8]. ASP (aka C3adesArg), a cleavage product of complement C3, promotes fatty acid uptake and glucose transport in adipose tissue; effects which are mediated by C5L2 [8] [9]. In C3 knockout mice, which are obligatorily ASP deficient, and C5L2 knockout (C5L2KO) mice, the absence of a functional ASP-C5L2 pathway leads to delayed postprandial triglyceride (TG) clearance and glucose transport, resulting in elevated concentrations in circulation for a longer time period [10] [11]. Moreover, our previous studies demonstrated that deficiency of C5L2 receptor altered metabolism and function in multiple tissues. We have previously shown decreased adipose tissue TG synthesis and increased muscle fatty acid oxidation in C5L2KO mice [11] [12]. Further, C5L2 receptor disruption increased circulating and adipose tissue inflammatory-related cytokines and enhanced diet-induced insulin resistance [7] [13].

It is well accepted that C5L2 also acts as a receptor for C5a, a potent anaphylatoxin, which also binds to the receptor C5aR (CD88) increasing chemotaxis in leukocytes and in vivo inflammatory response [14]. However, the functional consequences of C5a/C5L2 interaction remain a controversial issue. Previous studies on C5L2KO mice have demonstrated both anti- and pro-inflammatory roles for C5L2 in mediating inflammation [15] [16]. C5L2 negatively regulates C5a-C5aR mediated interaction in contact sensitivity models [16]. In contrast, C5L2 plays a pro-inflammatory role in a murine model of acute experimental colitis [15].

Recently, roles for C5L2 and C5a/C5aR in energy metabolism and inflammation have been demonstrated. We recently showed that C5L2KO mice on diet-induced obesity (DIO) treatment were less insulin sensitive than wildtype mice, due to increased inflammation and alterations in energy metabolism that led to ectopic fat deposition. More specifically, hepatic glucose uptake and oxidation were significantly increased, in C5L2KO mice vs wildtype controls, while fatty acid oxidation was decreased and liver triglyceride content increased [13]. Furthermore, macrophage infiltration was increased in the liver of C5L2KO mice [13], suggesting a pro-inflammatory effect stemming from C5L2 disruption. Although the study focused on insulin resistance, the described phenotype of C5L2KO mice on a DIO regimen clearly suggests that the disruption of C5L2 could lead to increased atherosclerosis as well. In that line of thought, increasing evidence supports complement activation association with atherosclerosis. Recent studies showed C5a and C5aR colocalized in human coronary atherosclerotic plaques [17] and elevated circulating C5a levels in patients with advanced atherosclerosis are considered as a predictor of adverse cardiovascular events [18]. Even more striking is the observation that C5a induces apoptosis of smooth muscle and endothelial cells [19]. This apoptosis is a well-known activator of an intense inflammatory response which further deteriorates plaque stability [20].

Strong links between atherosclerosis and non-alcoholic steatohepatitis [21] suggest a role for liver in the progression of atherosclerosis. A recent study demonstrated that liver could participate in the process of atherosclerosis [22]. Further, high fat/high cholesterol diets were associated with triggering inflammatory responses in the liver and aorta of ApoEKO mice, suggesting a chronological and quantitative relationship between liver impairment and the formation of atherosclerotic lesions [23]. However, to date, there is a lack of information concerning the role of C5L2 in liver and in atherosclerosis progression. Although a previous study showed decreased expression of C5L2 in aortas and liver of ApoEKO mice [24], the contribution of C5L2 to the development of atherosclerosis is still unknown.

The aim of the present study was to further analyze the contribution of C5L2 to the development of atherosclerosis. We first examined C5L2KO mice fed chow or DIO regimens and assessed factors linked with atherosclerosis that were not scrutinized in the past, focusing greatly on liver metabolism. Then we proceeded to utilize a new animal model that was both C5L2 and ApoE deficient (2KO) in order to fully comprehend the role of C5L2 in plaque development, as ApoEKO mice readily develop atherosclerosis in a short time frame, making it possible to see plaque formation in this animal model. These mice were fed chow or DIO diets as well and were examined for atherosclerotic plaque development, energy metabolism and inflammatory parameters. We hypothesized that, with DIO feeding, C5L2 deficiency would promote a phenotype that encourages atherosclerosis development. Coupled to ApoE deficiency, 2KO mice would show exacerbated atherosclerotic plaque formation.

2. Methods

2.1. Mice and Diets

All studies were conducted in accordance with the CCAC (Canadian Council on Animal Care guidelines). All protocols were approved by the Laval University Animal Care Committee. C5L2KO mice were obtained and genotyped as previously described [11]. The ApoEKO mice were obtained from Jackson Labs and crossed in-house with C5L2KO mice to generate C5L2/ApoE double knockout (2KO) and ApoEKO single knockout controls. All mice were on a C57Bl/6 background and had been backcrossed for at least eight generations. All mice were housed in a sterile barrier facility with a 12 h light: 12 h dark cycle.

At 8 - 10 weeks, mice were place on a diet inducing obesity (DIO) or maintained on chow diet (18% kcal from fat) for 12 - 15 weeks, as indicated. C5L2KO and WT mice on DIO were fed a high fat-high sucrose; 58% kcal fat (D12331, Research Diets Inc., New Brunswick, NJ, USA) as used previously with this model [13]. ApoE/C5L2 2KO and ApoE KO mice on DIO were placed on a more atherogenic diet (high fat-high sucrose; 41% kcal from fat, D12079B, Research Diets Inc., New Brunswick, NJ, USA) which contains an added 0.21% cholesterol to further induce atherosclerosis.

After 12 h fasting, mice were euthanized and samples collected for analysis. Blood samples were collected through cardiac puncture and tissues were collected, weighed and immediately frozen in liquid nitrogen. Tissues were subsequently transferred to −80˚C until analysis.

2.2. Liver Glycogen Content

Liver glycogen was measured in 30 - 50 mg of liver pieces as glucose units following acid hydrolysis of glycogen using 1 M HCl at 100˚C for 3 h. The extracts were neutralized with 2 M TRIS-KOH and the supernatant was assayed for glucose. Results are expressed as μmoles of glucose per gram (g) of liver wet weight.

2.3. Glucose Oxidation

Glucose oxidation was measured ex vivo in liver pieces as previously described [25] with the following modification: benzethonium hydroxide was added as the CO_2 trapping agent, rather than KOH. Results are expressed as pmoles of oxidized glucose per g of liver protein content. Recombinant human ASP/C3ades-Arg was prepared and purified by a modification of the original procedure [26], using a His-tag at the amino-terminal, with initial purification on a Ni-Sepharose column. His-tag was consequently removed by enterokinase reaction and further purified by HPLC. No denaturing agents were used at any step in the purification to avoid ASP inactivation.

2.4. Liver Enzyme Assays

Liver samples were maintained frozen at –80˚C until assayed for maximal (Vmax) enzyme activities. Approximately 250 mg of liver was homogenized with a glass-on-glass homogenizer in ice cold buffer (1:20 weight: volume) (0.1 M phosphate buffer with 1 mM PMSF, 2 mM EDTA, 1 mM DTT, 10% glycerol, 0.25 M sucrose). Half of the homogenate was aliquoted and frozen at –80˚C for future determination of glycogen phosphorylase (PHOS), hydroxyacyl-CoA dehydrogenase (HADH) and fructose 1,6-biphosphatase (F1,6-BP) maximal activities, as previously described [25] [27]. The other half of the homogenate was ultracentrifuged at 105,000 g for 1 h at 4˚C to isolate crude microsomes. The resulting pellet was resuspended in ice-cold buffer (1:10 weight: vo-

lume) and was used for measurement of diacylglycerol acyltransferase 1 and 2 (DGAT1, DGAT2) and glucose 6-phosphatase (G6Pase) activities, as described [28].

2.5. Liver Lipid Content

Liver neutral lipids (cholesterol ester (CE), triglyceride, and non-esterified fatty acids (NEFA) were extracted overnight at 4˚C from 30 - 50 mg of liver tissue using heptane:isopropanol (3:2). The extract was transferred while the remaining tissue was air dried, dissolved in 0.3 N NaOH and assessed for protein content using the Bradford method (Bio-Rad, Mississauga, ON, Canada). Organic extracts were lyophilized and lipids were re-dissolved in 10% Triton X-100 aqueous solution. TG, NEFA and CE were measured using commercial colorimetric kits. Results are expressed as μmoles of TG, NEFA or CE per gram (g) of protein.

2.6. VLDL-TG Secretion

Prior to initiation of DIO feeding, mice were fasted for 2 hours and then injected with 20 mg of tyloxapol (Triton WR1339, Sigma) in 100 μL of sterile saline through the tail vein. Blood samples were drawn before the injection and at 30, 60, 90 and 120 minutes post-injection by saphenous or submandibular vein bleeding. The same procedure was repeated after 4 weeks of DIO feeding. Plasma TG was measured in each sample. The linear portion of the regression of plasma TG concentration as a function of time was used for statistical analysis.

2.7. Plasma Ketone Body 3-Hydroxybutyrate (3-HB)

After 12 weeks of chow or DIO diet, plasma 3-HB was measured using colorimetric enzymatic kits (Wako Autokit 3-HB) as per the manufacturer's instructions. Results are expressed as μmoles/L.

2.8. Real-Time Quantitative RT-PCR

Wildtype and C5L2KO mice liver tissue mRNA was extracted, purified and reverse transcribed into cDNA using RNeasy Mini kits or RNeasy Lipid T issue Mini kit and Quantitect Reverse Transcription kits (Qiagen, Gaithersburg, MD, USA). mRNA for the housekeeping gene glyceraldehyde dehydrogenase (GAPDH) and acetyl-CoA carboxylase alpha (ACACA) were measured using QuantiTect Primer Assays (Qiagen, Gaithersburg, MD,USA). mRNA for Interleukin-6 (IL-6), Keratinocyte-derived Chemokine (KC), Monocyte Chemotactic Protein-1 (MCP-1), Macrophage Inflammatory Protein-1α (MIP-1α), Plasminogen Activator Inhibitor-1 (PAI-1) and Tumor Necrosis Factor-α (TNF-α) was quantified using custom primers designed through NCBI Mouse Primer Depot (http://mouseprimerdepot.nci.nih.gov/). Primer sequences were as follows:IL-6 forward GAGGATACCACTCCCAACAGACC; IL-6 reverse AAGTGCATCATCGTTGTTCATACA; KC forward TCTCCGTTACTTGGGGACAC; KC reverse CCACACTCAAGAATGGTCGC; MCP-1 forward ATTGGGATCATCTTGCTGGT; MCP-1 reverse CCTGCTGTTCACAGTTGCC; MIP-1 α forward GTGGAATCTTCCGGCTGTAG; MIP-1 α reverse ACCATGACACTCTGCAACCA; PAI-1 forward GCCAGGGTTGCACTAAACAT; PAI-1 reverse GCCTCCTCATCCTGCCTAA; TNF-α forward CATCTTCTCAAAATTCGAGTGACAA; TNF-α reverse TGGGAGTAGACAAGGTACAACCC. Relative gene expression was calculated and corrected using GAPDH as the housekeeping gene. All procedures followed manufacturer's instructions and MIQE guidelines [29].

2.9. Plasma Analysis

Following diet regimen, ApoEKO and C5L2/ApoE-2KO mice were fasted for 12 hours and then euthanized. Fasting plasma samples were taken by cardiac puncture. Plasma cholesterol (CHOL), TG, NEFA and glucose were measured using colorimetric enzymatic kits as follows: plasma CHOL and TG (Roche Diagnostics, Richmond, VA, USA), NEFA (Wako Chemicals, Richmond, VA, USA) and glucose (Sigma, Saint Louis, MO, USA). Inflammatory cytokines IL-6, KC, MCP-1, were measured using suspension bead array immunoassay kits following the manufacturer's specifications (Bio-Plex Pro Mouse Cytokine Assay 23-plex, Biorad, Mississauga, ON, Canada) on a Bio-Plex series 100 instrument (BioRad, Mississauga, ON, Canada), and information on intra- and inter-assay CVs is provided by the manufacturer and is freely available.

2.10. Glucose Tolerance Test

Glucose tolerance test (GTT) was performed on ApoEKO and C5L2/ApoE-2KO mice following 12 hour fast after DIO or chow diet regimen. Blood samples were taken by saphenous vein bleeding at 0, 30, 60 and 90 min after an intraperitoneal glucose injection (2 g/kg body weight). Glucose was measured using colorimetric enzymatic kits (Sigma).

2.11. Atherosclerosis Oil Red O Staining and Quantification

At the end of diet treatment, ApoEKO and C5L2/ApoE-2KO mice were fasted for 12 hours and then euthanized. The thorax was opened and fat and tissue around the ascending aorta, brachiocephalic artery and heart were removed. Hearts and aorta were flushed with phosphate buffered saline (PBS).

For "en face" analysis, the whole aortae were isolated, and stored in 4% PFA (4% w/v paraformaldehyde in PBS) at 4˚C, transferred to PBS after 24 hours and then stained with Oil Red-O. Aortas were rinsed in ddH_2O followed by 60% isopropanol for 2 minutes and then immersed in Oil Red-O for 10 - 15 minutes. Aorta were then rinsed in 60% isopropanol for 2 minutes, followed by ddH_2O, and stored in ddH_2O at 4˚C until pinning out and photographed. The surface area occupied by atherosclerosis was quantified by the "en face" method at the aortic arch and major branches, including the brachiocephalic, carotid, and subclavian arteries with ImagePro software (Media Cybernetics Inc, Rockville, MD).

2.12. Statistical Analysis

Results are expressed as mean ± standard error of the mean (SEM) in the figures and as mean ± standard deviation (SD) in the table and text as indicated. Groups were compared with linear regression, ANOVA or T-test using Prism software (Graphpad Prism 5, San Diego, CA, USA). Statistical significance was set as $p < 0.05$, where NS indicates not significant.

3. Results

3.1. Changes in Glucose Metabolism in the Liver of C5L2KO Mice

As shown in **Figure 1(A)**, liver glycogen content was reduced in C5L2KO (−34%, $p < 0.05$) mice following chow diet compared to their wildtype (WT) counterparts. PHOS, which is responsible for glycogen degradation into glucose 6-phosphate monomers, was increased in chow diet (+40% C5L2KO $p = 0.059$). Glucose-6-phosphatase (G6Pase) activity, which controls G6P dephosphorylation for release of glucose, was also increased on a chow diet (+34% C5L2KO $p < 0.05$ **Figure 1(A)**). With DIO feeding, glycogen content was decreased compared to WT chow diet, but C5L2KO mice were higher (+51%, $p < 0.05$) relative to WT DIO (**Figure 1(B)**). However, there were no significant differences in PHOS and G6Pase activities between C5L2KO and WT mice with DIO feeding (**Figure 1(B)**). Fructose 1,6-biphosphate (F1,6-BP) activity, the rate limiting step in glucose oxidation, as well as a direct evaluation of ex vivo glucose oxidation were assessed (**Figure 1(C)**). F1,6-BP activity in C5L2KO mice was comparable to WT mice with both chow and DIO regimens. In C5L2KO mice, ex vivo hepatic glucose oxidation was significantly increased (+90%, $p < 0.001$ **Figure 1(D)** as was previously demonstrated). Addition of rASP [100 nM] significantly decreased ex vivo glucose oxidation in WT hepatic tissue (−20%, $p < 0.01$) while it had no effect on C5L2KO hepatic tissue, which is lacking the ASP receptor (**Figure 1(D)**).

3.2. Altered Hepatic Fatty Acid Oxidation and Lipid Content in C5L2KO Mice

In order to evaluate fatty acid metabolism in the liver, the following parameters were assessed: fasting lipid content, enzymatic activities and in vivo lipoprotein secretion. On a chow diet, hepatic TG content of C5L2KO mice was comparable to WT controls. With DIO feeding, liver TG was higher in C5L2KO mice (+49%, $p < 0.01$), in addition to the expected increase when compared to chow-fed animals (**Figure 2(A)**). Fasting hepatic NEFA content of C5L2KO mice followed the same trend, as it remained unchanged between KO and WT on a chow diet but was increased in C5L2KO mice fed a DIO regimen (+34%, $p < 0.05$) compared to WT controls (**Figure 2(A)**). Cholesterol ester content of the liver remained unchanged when compared to WT in C5L2KO mice on both diets (data not shown). ACACA, the rate limiting step enzyme in fatty acid synthesis, had signifi-

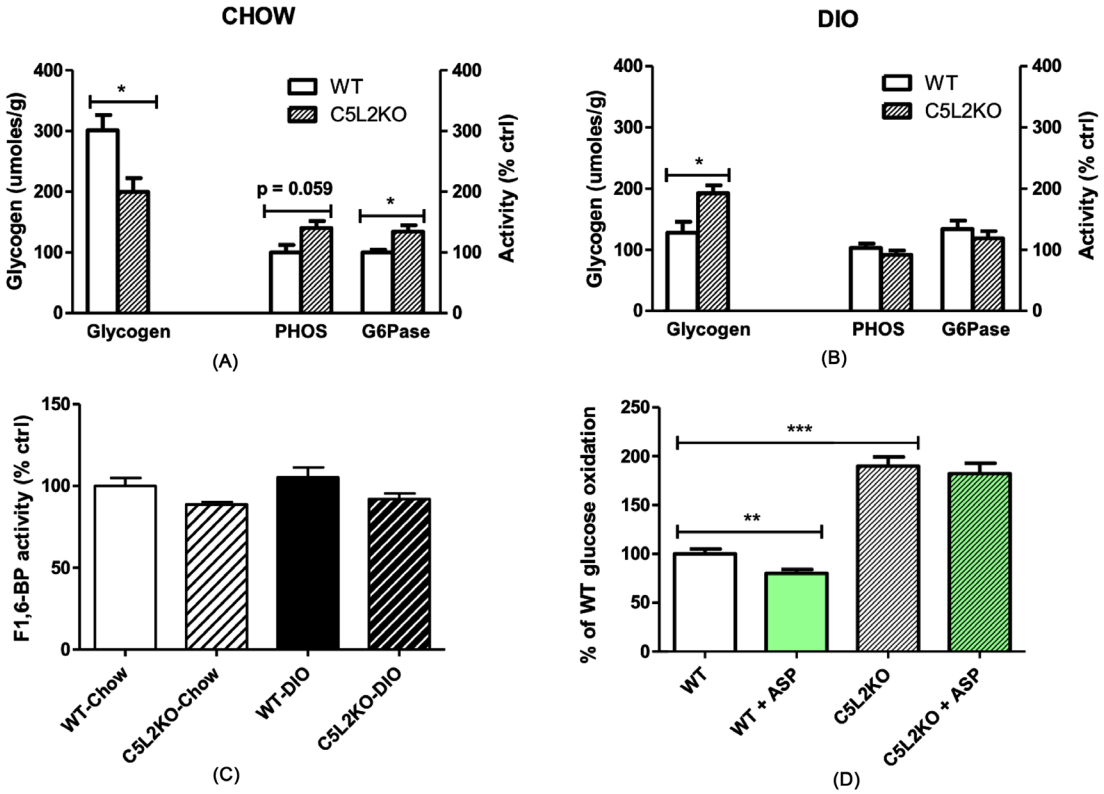

Figure 1. Hepatic glucose metabolism in C5L2KO and WT mice. Hepatic glycogen content (panels (A) and (B)), glycogen phosphorylase (PHOS) activity (panels (A) and (B)), and glucose 6-phosphatase (G6Pase) activity (panels (A) and (B)) with chow or DIO diets are shown. Fructose 1,6-biphosphatase (F1,6-BP) enzyme activity (panel (C)) was measured in tissue homogenates, and ex vivo hepatic glucose oxidation with and without ASP treatment was measured in tissue explants from mice (panel (D)). Results are expressed as glycogen-derived glucose per liver wet mass (μmoles/g) or activity relative to chow fed WT controls (% of ctrl) expressed as mean ± SEM where differences vs WT controls are expressed as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

cantly higher gene expression in C5L2KO mice vs WT mice on DIO diet (**Figure 2(B)**).

Diacylglycerol acyltransferase, the final step in triglyceride synthesis, is expressed in two forms in the liver, DGAT1 and DGAT2. It has been suggested that TG synthesized via DGAT1 are primarily used for lipoprotein secretion, while DGAT2-derived TG are more likely to be stored [30] [31]. As shown in **Figure 2(C)**, DGAT2 activity was decreased in C5L2KO vs WT mice on a chow diet. With DIO feeding, DGAT2 activity tended to be decreased in C5L2KO mice, although not significant. By contrast, DGAT1 activity was found to be increased in the C5L2KO mice on chow diet (+36%, $p < 0.05$), while no change was seen in DIO animals (**Figure 2(D)**).

In spite of increased hepatic lipid content, C5L2KO mice showed a normal hepatic VLDL-TG secretion rate when compared to WT on a chow diet (0.145 ± 0.01 WT vs 0.135 ± 0.01 C5L2KO mmol/L·min, respectively, **Figure 2(E)**, pNS), in line with DGAT1 activity. Following DIO feeding, TG secretion rates also remained comparable in C5L2KO vs WT (0.117 ± 0.01 WT vs 0.108 ± 0.01 C5L2KO mmol/L·min, respectively, **Figure 2(F)**, pNS).

Beta-oxidation rates in liver mitochondria (measured using hydroxyacyl-CoA dehydrogenase maximal activity) were reduced in C5L2KO mice with both chow and DIO regimen (**Figure 2(G)**, −35% C5L2KO on chow $p < 0.05$, −42% C5L2KO on DIO, compared to their respective WT controls). Ketone bodies (3-hydroxybutyric acid, 3-HB) were increased in C5L2KO mice fed a chow diet compared with WT controls ($p < 0.01$) (**Figure 2(H)**). However, the 3-HB level was similar in the two groups following DIO feeding (**Figure 2(H)**).

3.3. The Liver of C5L2KO Mice Is a Significant Source of Pro-Inflammatory Cytokines

Since liver inflammation is linked with atherosclerosis and C5L2KO mice have been demonstrated to have in-

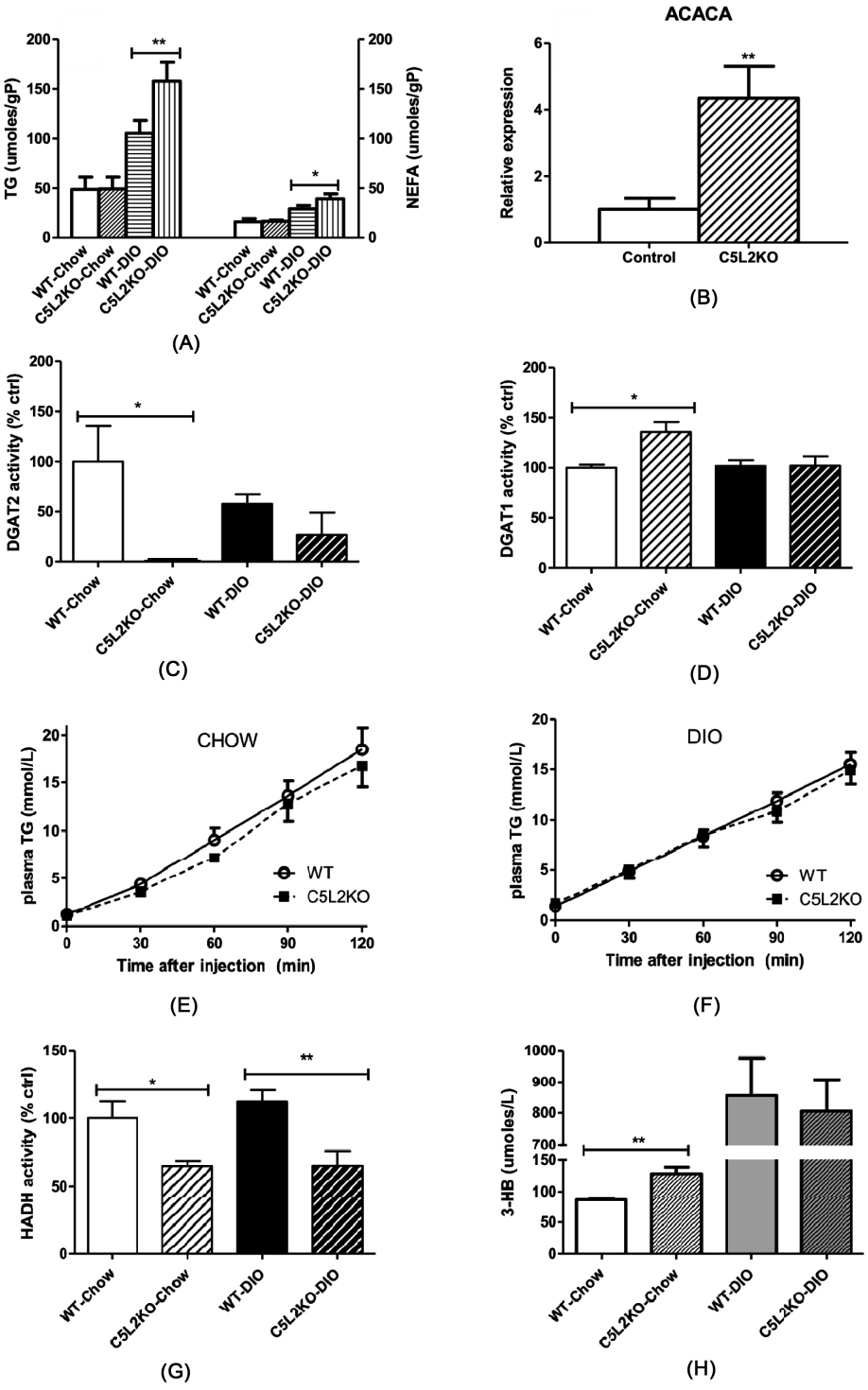

Figure 2. Hepatic lipid metabolism in C5L2KO and WT mice. Hepatic triglyceride (panel (A)) and non-esterified fatty acids (NEFA) (panel (A)) content, hepatic ACACA gene expression analysis (panel (B)), hepatic diacylglycerol acyltransferase 2 (DGAT2) activity (panel (C)) and DGAT1 activity (panel (D)), VLDL TG secretion (panels (E) and (F)), hydroxyacyl-CoA dehydrogenase (HADH) activity (panel (G)) and ketone bodies3-hydroxybutyric acid (3-HB) (panel (H)) are shown. Results are expressed as lipid content per cell protein (μmoles/gP) (panel (A)), relative gene expression (panel (B)), activity relative to chow fed WT controls (% of ctrl) (panels (C), (D) and (G)), plasma TG (mmoles/L) (panels (E) and (F)) or plasma 3-HB (umoles/L) (panel (H)). Results are expressed as mean ± SEM where differences vs WT controls are expressed as $^{*}p < 0.05$, $^{**}p < 0.01$.

creased circulating pro-inflammatory cytokines, liver pro-inflammatory state in C5L2KO mice vs wildtype was evaluated. Gene expression of several inflammatory cytokines was measured in the liver. With DIO feeding, the expression of inflammation-related cytokines, including IL-6, KC, MIP-1α and PAI-1, were significantly increased in the liver of C5L2KO mice compared to wildtype mice (**Figure 3**) while levels of hepatic TNF-α and MCP-1 tended to be elevated, but not significantly (**Figure 3**).

3.4. Characterization of the ApoE/C5L2-2KO Model

The changes in liver inflammation and lipid metabolism observed in C5L2KO animals point toward elevated risks for the development of atherosclerosis. However, as rodent models have a short lifespan and are not prone to develop atherosclerosis naturally, we used an atherosclerosis-prone model, the ApoEKO mouse, and crossed it with C5L2KO mice to further evaluate how the disruption of C5L2 could affect atherosclerosis development. C5L2/ApoE-2KO and ApoEKO mice controls were fed DIO or chow diets for 15 weeks. Fasting cholesterol levels were significantly increased with DIO treatment compared to mice on chow diet in both ApoEKO and C5L2/ApoE-2KO groups (**Figure 4(A)**), reflecting the high cholesterol content of the diet. Cholesterol concentrations were also significantly higher in the C5L2/ApoE-2KO mice vs ApoEKO control with DIO treatment (**Figure 4(A)**). However, no significant change was observed in plasma glucose, TG or NEFA comparing C5L2/ApoE-2KO mice to ApoEKO controls on any diet, although a trend for elevated plasma triglycerides was noted (**Table 1**). An intraperitoneal glucose tolerance test (GTT) was performed with glucose levels monitored throughout a 90-minute time frame after the injection. C5L2/ApoE-2KO mice had significantly delayed glucose clearance vs ApoEKO mice on both chow diet ($P < 0.0001$, **Figure 4(B)**) and DIO feeding ($P = 0.0026$, **Figure 4(C)**).

3.5. Effects of C5L2 Deficiency on Plasma Inflammatory Cytokines in ApoE/C5L2-2KO Mice

Plasma inflammatory cytokines were measured in C5L2/ApoE-2KO and ApoEKO mice after chow or DIO regimen. The expression of IL-6 was significantly higher in C5L2/ApoE-2KO compared to ApoEKO mice with chow diet (**Figure 4(D)**). The same trend for IL-6 was seen with DIO feeding, although it did not reach significance. There was no difference in KC and MIP-1α levels between C5L2/ApoE-2KO and ApoEKO mice with chow or DIO treatment (**Table 1**).

3.6. C5L2 Deficiency Increases Atherosclerotic Plaques in ApoE/C5L2-2KO Mice

Lesion areas in the aorta were assessed after chow or DIO regimen in C5L2/ApoE-2KO and ApoEKO mice. Plaque area, assessed at multiple sites along the vessels, was significantly and strongly increased in C5L2/ApoE-2KO mice compared with ApoEKO controls after both chow diet ($p < 0.05$) or DIO ($p < 0.001$). Lesion

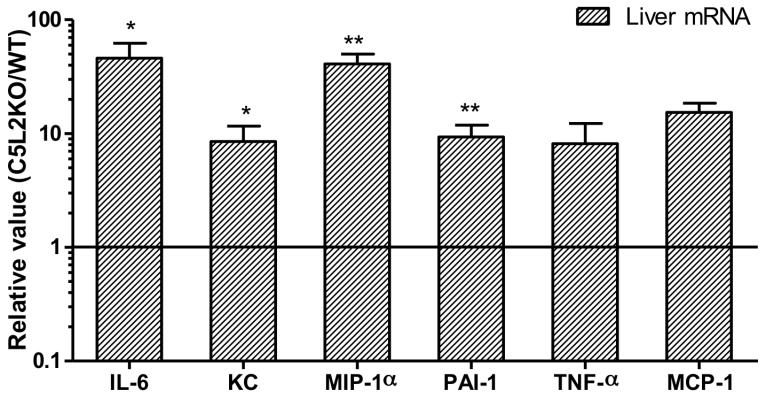

Figure 3. Hepatic inflammatory cytokine mRNA expression in C5L2KO and WT mice. Gene expression analysis of liver inflammatory cytokines is shown in **Figure 3**. Results are expressed as relative gene expression mean ± SEM where differences vs WT controls are expressed as $^{*}p < 0.05$, $^{**}p < 0.01$.

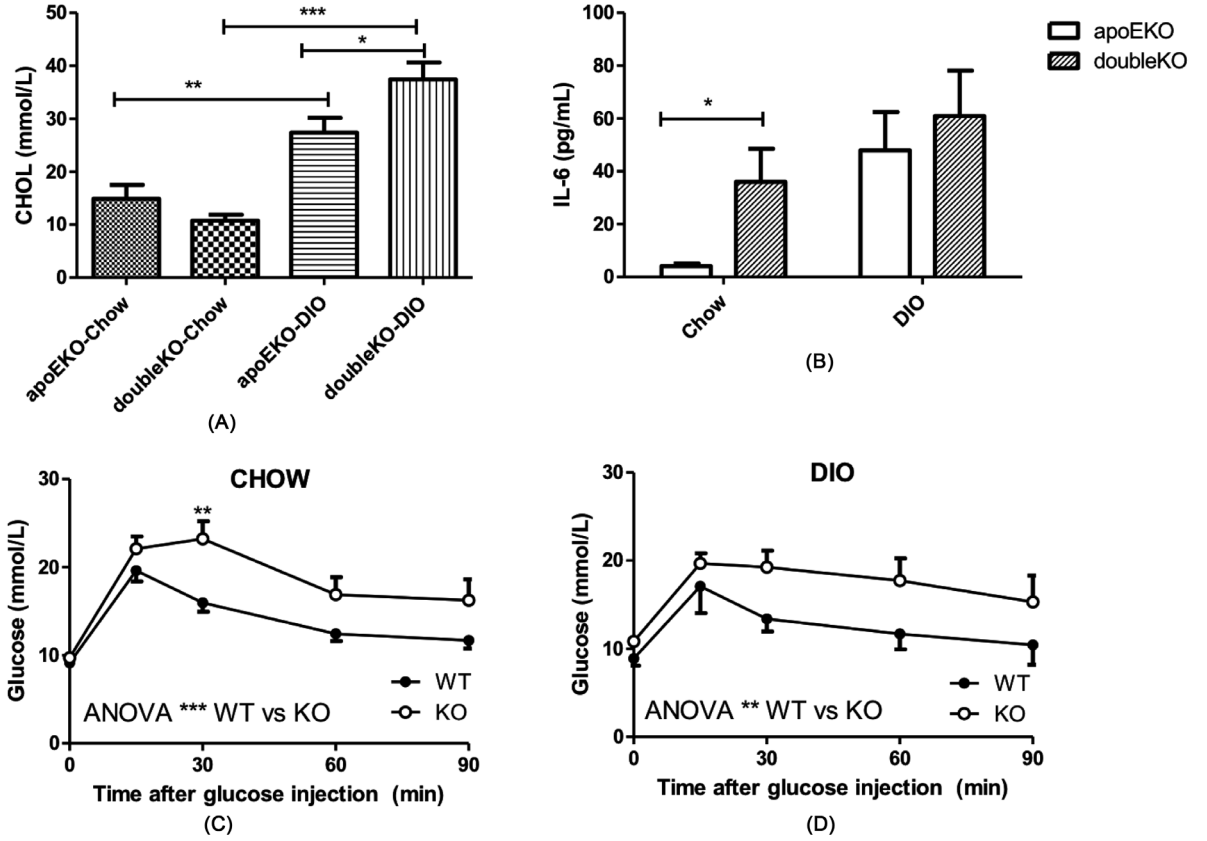

Figure 4. Plasma lipid concentration, GTT and inflammatory cytokines in C5L2/ApoE-2KO and ApoEKO mice. Plasma cholesterol concentration (panel (A)), glucose tolerance tests (panels (B) and (C)) and plasma IL-6 (panel (D)) are shown. Results are expressed as mmol/L (panels (A)-(C)) and pg/mL (panel (D)). Results are expressed as mean ± SEM where differences vs ApoEKO controls are expressed as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Table 1. C5L2/ApoE-2KO and ApoEKO mice plasma parameters. Fasting plasma parameters of C5L2/ApoE-2KO mice vs ApoEKO controls. ApoEKO and C5L2/ApoE-2KO on each diet were compared by t-test. The abbreviations used are: DIO: diet-induced obesity; KC: Keratinocyte-derived Chemokine; KO: knockout; MIP: Macrophage Inflammatory Protein; NEFA: non-esterified fatty acids; TG: triglyceride.

Parameter\Diet	**Chow**			**DIO**		
Genotype	ApoEKO	C5L2/ApoE-2KO	p value	ApoEKO	C5L2/ApoE-2KO	p value
TG (mmol/L)	1.40 ± 0.38	1.55 ± 0.87	0.65	1.09 ± 0.50	1.64 ± 0.86	0.14
NEFA (mmol/L)	0.49 ± 0.11	0.57 ± 0.17	0.24	0.38 ± 0.10	0.46 ± 0.14	0.20
Glucose (mmol/L)	16.77 ± 4.70	12.20 ± 4.42	0.07	15.38 ± 3.13	17.45 ± 5.98	0.29
KC (pg/mL)	206.2 ± 196.3	190.3 ± 244.1	0.88	123.6 ± 75.62	143.7 ± 13.9	0.37
MIP (pg/mL)	43.36 ± 106.2	187.2 ± 341.3	0.26	55.55 ± 91.29	83.57 ± 99.94	0.55

area also correlated, in both groups, with DIO regimen (**Figure 5**).

4. Discussion

It is now well accepted that atherosclerosis is a result of a combination of lipid storage and inflammation although the mechanisms are not completely clear. C5L2 has been demonstrated to play a dual role in metabolism and immunity [7]. In the present study, we evaluated the effects of C5L2 deficiency on lipid/glucose metabolism and inflammation in different knockout mice models, first using C5L2 KO as compared to WT mice and then to

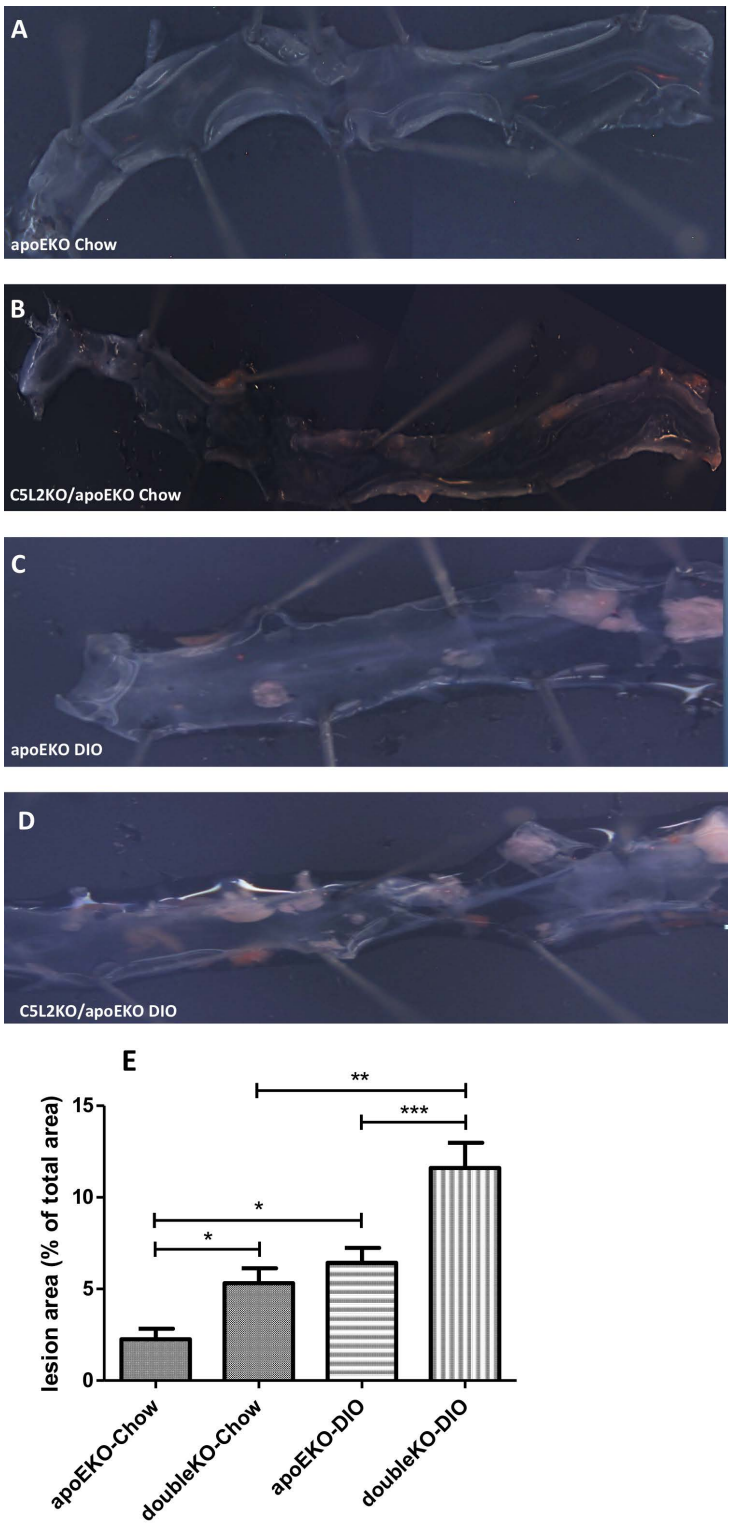

Figure 5. Atherosclerotic lesion area in the aortas. Aortic sections were collected and stained with oil red-O. Lesions are quantified using computer assisted densitometric analysis. Representative oil red-O stained images from C5L2/ApoE-2KO and ApoEKO mice following chow and DIO diets are shown on panels (A)-(D) (Magnification 8X). Quantification of aorta lesion area in C5L2/ApoE-2KO and ApoEKO mice is expressed as % lesion area/total area scanned and is shown on panel E. Results are expressed as mean ± SEM where differences vs ApoEKO controls are expressed as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

investigate in further detail atherosclerotic plaque formation using a dual ApoE and C5L2 deficient animal model as compared to ApoE KO. We thus confirmed the susceptibility of C5L2 deficient mice to atherosclerosis using a novel animal model, the ApoE-C5L2-2KO mice. On both chow and DIO regimens, a significant increase in atherosclerotic plaque area was detected, suggesting that C5L2 deficiency does lead to increased atherosclerosis.

According to the novel findings shown here and integrating what is known in the literature, we believe that several evidences point toward a functional role for C5L2 in atherosclerosis development. First, C5L2 deficiency alters glucose metabolism and enhances insulin resistance. Hepatic glucose oxidation is significantly increased in C5L2KO mice vs WT, which might be due to the absence of ASP inhibition of glucose oxidation in C5L2KO, an effect which is present in WT mice (but not observed in C5L2KO since the ASP signaling pathway is disrupted). This increased oxidation is also accompanied by increased glycogen content upon DIO feeding in the C5L2KO mice.

Furthermore, notwithstanding similar fasting glucose levels, a significantly delayed glucose clearance is still manifested in C5L2/ApoE-2KO mice vs ApoEKO mice on both diets. In order to maintain similar glucose levels, a higher insulin level is needed to compensate [13], suggesting increased insulin resistance in C5L2/ApoE-2KO mice. A high insulin level is well-known to promote atherosclerosis development [32]. Second, C5L2 deficiency profoundly alters whole-body lipid metabolism. C5L2 deficient animals have been shown to exhibit a delay in postprandial triglyceride clearance [8], and increased ectopic lipid deposition [11] [13] both of which might contribute to the development of atherosclerosis. Because the functional ASP-C5L2 pathway is impaired in C5L2KO mice, TG synthesis in adipose tissue is decreased [11]. When C5L2KO mice were fed a DIO regimen, excess lipid overflows into other tissues or organs and results in lipid redistribution [12] [13]. Here, increased liver fatty acid synthesis and glycogen content are noted concurrently with increased glucose oxidation when C5L2KO mice are in an obese state. Taken together, redistribution of lipids in excess of liver oxidation capacities likely explains the increase in ectopic lipid deposition. Consequently, hepatic TG and NEFA content are significantly increased in C5L2KO mice with DIO feeding.

Ectopic lipid deposition, particularly in the liver, is detrimental for plaque formation and is most likely an early effector in the atherosclerotic development pathway. This would be achieved through elevated levels of circulating inflammatory mediators as well as various oxidative stress markers and through abnormal postprandial lipoprotein metabolism [33]. In ApoE/C5L2-2KO mice, plasma cholesterol was increased as well, further aggravating the phenotype. Previous studies show that hypercholesterolemia plays a pivotal role in the initiation of atherosclerosis [34] with cholesterol crystals in atherosclerotic lesions inducing complement activation and increased release of inflammatory cytokines, contributing to the progression of atherosclerosis [35].

Further, C5L2 deficiency modulates inflammation in the liver and in circulation, which could also contribute to the development of atherosclerosis. A previous study showed that C5L2KO mice exhibited a more pronounced inflammatory state than WT controls, with increased M1 macrophage infiltration within the liver and elevated plasma concentrations of several pro-inflammatory cytokines [13]. We demonstrate in this study that the expression of hepatic IL-6, KC, MIP-1α and PAI-1 mRNA is significantly higher in C5L2KO mice than in their WT counterparts with DIO feeding and, therefore, that there is a strong local hepatic inflammation that contributes significantly to the whole-body pro-inflammatory phenotype. Accumulating evidence shows a strong association between atherosclerosis and non-alcoholic steatohepatitis (NASH) and the hepatic inflammatory response seems to be involved in the formation of early atherosclerotic lesions [21] [36].

As ASP has been implicated to play an important role in lipid metabolism, atherosclerotic susceptible mice that were deficient in C3, and therefore unable to synthesize ASP, were shown to be hyperlipidemic with increased atherosclerosis (these mice were dual ApoE and LDLR deficient) [37]. Accordingly, deficiency of C5L2 in ApoEKO mice, because of the impaired functional ASP-C5L2 pathway, could promote atherosclerosis and result in increased plaque area.

Further, the proinflammatory and proatherosclerotic effects of C5L2 disruption could stem from the following additional aspects. Firstly, the absence of C5a binding with C5L2 and lack of C5L2 and C5aR heterodimerization [38] may allow more C5a to be available for binding to C5aR, thereby accentuating its proinflammatory activity. In addition, the lack of ASP binding with C5L2 may result in a decreased conversion of C3a to ASP, which could allow more C3a to bind to C3aR, which is also a proinflammatory stimulant. C3a and C5a might promote infiltration of inflammatory cells into the plaque; it has been shown that the expression of C3aR and C5aR was significantly increased in human atherosclerotic coronary plaques [39]. It has also been shown that

treatment of C5a increased plaque disruption and plaque fibrin content in apoEKO mice [19]. Treatment of ApoEKO mice with C5aR antagonist reduced lipid content, inflammatory cell infiltration and lesion size in the plaque [24] [40].

5. Conclusion

In summary, we propose that deficiency of C5L2 disrupts both the ASP-C5L2 pathway and C5a-C5L2 interaction. Our study demonstrates that the disruption of C5L2 aggravates atherosclerosis, through worsened glucose and lipid metabolism, in addition to increased hepatic and circulating inflammation.

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Competing Interests

The authors have declared that no competing interests exist.

Author's Contributions

YL and AF carried out all of the experimental planning, assays, data analysis and initial manuscript drafting; ML participated in the design of the study, performed statistical analysis and writing of the manuscript; KC conceived of the study, participated in its design and coordination and manuscript writing. All authors read and approved the final manuscript.

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List of Abbreviations

ASP: Acylation Stimulating Protein;
CHOL: Cholesterol;
DIO: Diets Inducing Obesity;
F1,6-BP: Fructose 1,6-Biphosphatase;
G6Pase: Glucose 6-Phosphatase;
GTT: Glucose Tolerance Test;
HADH: Hydroxyacyl-CoA Dehydrogenase;
3-HB: 3-Hydroxybutyric Acid;
KO: Knockout;
NEFA: Non-Esterified Fatty Acids;
PBS: Phosphate Buffered Saline;
PHOS: Phosphorylase;
TG: Triglyceride;
WT: Wildtype.