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Chemokine-Like Receptor 1 Deficiency Does Not Affect the Development of Insulin Resistance and Nonalcoholic Fatty Liver Disease in Mice

Nanda Gruben1, Marcela Aparicio Vergara1, Niels J. Kloosterhuis1, Henk van der Molen1, Stefan Stoelwinder1, Sameh Youssef2, Alain de Bruin2, Dianne J. Delsing3, Jan Albert Kuivenhoven1, Bart van de Sluis1, Marten H. Hofker1,∗, Debby P. Y. Koonen1,∗*

1 University of Groningen, University Medical Center Groningen, Department of Pediatrics, Molecular Genetics Section, Groningen, the Netherlands, 2 Utrecht University, Faculty of Veterinary Medicine, Dutch Molecular Pathology Center, Utrecht, the Netherlands, 3 Merck Research Laboratories, MSD, Department of Immune Therapeutics, Oss, the Netherlands

Abstract

The adipokine chemerin and its receptor, chemokine-like receptor 1 (Cmklr1), are associated with insulin resistance and nonalcoholic fatty liver disease (NAFLD), which covers a broad spectrum of liver diseases, ranging from simple steatosis to nonalcoholic steatohepatitis (NASH). It is possible that chemerin and/or Cmklr1 exert their effects on these disorders through inflammation, but so far the data have been controversial. To gain further insight into this matter, we studied the effect of whole-body Cmklr1 deficiency on insulin resistance and NAFLD. In view of the primary role of macrophages in hepatic inflammation, we also transplanted bone marrow from Cmklr1 knock-out (Cmklr1−/−) mice and wild type (WT) mice into low-density lipoprotein receptor knock-out (Ldlr−/−) mice, a mouse model for NASH. All mice were fed a high fat, high cholesterol diet containing 21% fat from milk butter and 0.2% cholesterol for 12 weeks. Insulin resistance was assessed by an oral glucose tolerance test, an insulin tolerance test, and by measurement of plasma glucose and insulin levels. Liver pathology was determined by measuring hepatic inflammatory and fibrotic gene expression, immune cell infiltration, lipid accumulation or NAS. In line with this, we detected no differences in insulin resistance. In concordance with whole-body Cmklr1 deficiency, the absence of Cmklr1 in bone marrow-derived cells in Ldlr−/− mice did not affect their insulin resistance or liver pathology. Our results indicate that Cmklr1 is not involved in the pathogenesis of insulin resistance or NAFLD. Thus, we recommend that the associations reported between Cmklr1 and insulin resistance or NAFLD should be interpreted with caution.


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* E-mail: d.p.y.koonen@umcg.nl
* These authors contributed equally to this work.

Introduction

Obesity is accompanied by the development of the metabolic syndrome, a cluster of metabolic abnormalities, which includes low-grade inflammation, dyslipidemia and insulin resistance. Since nonalcoholic fatty liver disease (NAFLD) is the hepatic manifestation of the metabolic syndrome, its increasing prevalence follows the increasing rates of obesity seen worldwide. As a result, NAFLD has become one of the main causes of chronic liver disease in Western societies [1]. NAFLD describes a broad spectrum of liver diseases, ranging from simple steatosis (intrahepatic fat accumulation) to nonalcoholic steatohepatitis (NASH), fibrosis, and cirrhosis [2]. NASH can be distinguished from simple steatosis by the presence of inflammation. It is unknown how NAFLD develops or which factors provoke its progression into NASH.

Recently, an adipokine named chemerin has been implicated in the metabolic syndrome and the progression of NAFLD. Chemerin was first identified as a chemo-attractant protein, attracting immune cells expressing the chemerin receptor chemokine-like receptor 1 (Cmklr1, also known as ChemR23) [3]. This suggests that chemerin has a pro-inflammatory role. However, studies investigating the role of Cmklr1 in inflammation are controversial. Cmklr1 knock-out (Cmklr1−/−) mice have been reported to be protected against central nervous system inflammation [4], but they were more susceptible to lipopolysaccharide-induced lung inflammation [5] and viral pneumonia [6]. As
inflammation is thought to play a key role in the progression of insulin resistance and NAFLD, chemerin and Cmklr1 may be involved in these disorders. Indeed, in several human populations, elevated plasma chemerin levels correlate positively with characteristics of the metabolic syndrome, including inflammation, insulin resistance, plasma lipids and body mass index [7–9]. In addition, elevations in serum chemerin levels were found in patients with NAFLD and NASH compared to healthy controls [10] and have been shown to positively correlate with markers of liver pathology, including fibrosis, portal inflammation and NAFLD activity score (NAS) [11]. In contrast, expression of chemerin in visceral adipose tissue in morbidly obese individuals correlated negatively with hepatic inflammation (unpublished data). Moreover, data on hepatic expression of chemerin and Cmklr1 in human and mouse NAFLD are inconsistent, since both reduced and increased levels of these genes have been found [12–14]. Although these studies indicate that chemerin and its receptor may be involved in the pathogenesis of insulin resistance and NAFLD, a causative role remains to be established.

Thus far, only a few studies have investigated the effect of Cmklr1 deficiency in metabolic disease and, similar to the studies investigating inflammation, the results have been controversial. In one study it was shown that Cmklr1 deficiency induced glucose intolerance in mice [15], whereas another study showed no effect of Cmklr1 deficiency on glucose intolerance [16]. In addition, Cmklr1−/− mice were found to have reduced hepatic steatosis on a low fat diet, but not on a high fat diet, whereas hepatic inflammation was reduced in Cmklr1−/− mice on both diets [15]. However, differences in inflammation were not confirmed by histological analyses and differences in body weight gain could not be excluded as a confounding factor in this study [15]. Moreover, despite the controversial role of Cmklr1 deficiency in inflammation, the role of Cmklr1 in NASH has not been investigated. In the current study, we investigated the effect of whole-body Cmklr1 deficiency on insulin resistance and NAFLD. In view of the primary role of macrophages in NASH, we also transplanted bone marrow from Cmklr1−/− mice and wild type (WT) mice into low-density lipoprotein receptor knock-out (Ldlr−/−) mice. These mice develop hepatic inflammation when fed a high fat, high cholesterol (HFC) diet and can be regarded as a mouse model for NASH.

Materials and Methods

Ethics Statement

The University of Groningen ethics committee approved all the animal experiments described in this paper (permit numbers 5964A and 5964E) and the animals’ discomfort was kept to a minimum.

Mice and Bone Marrow Transplantation

Male mice deficient for Cmklr1 on a C57BL/6J background and wild type (WT) littermates were kindly provided by MSD (Oss, the Netherlands) and kept on a 12-hour light/12-hour dark cycle, with ad libitum access to food and water. Starting at 3–4 months of age, they were fed a high fat, high cholesterol (HFC) diet, containing 21% fat from milk butter and 0.2% cholesterol (Scientific Animal Food and Engineering, Villemoisson-sur-Orge, France), for 12 weeks to induce NAFLD. To investigate the loss of Cmklr1 in bone marrow-derived cells, female low-density lipoprotein receptor knock-out (Ldlr−/−) mice on a C57BL/6J background (bred in-house) were irradiated with 9.5 Gy [17] using X-Rad 320 (Precision X-ray, CT, USA) at 2–5 months of age. The next day, these mice were transplanted with bone marrow from WT or Cmklr1−/− mice by intravenous injection. To allow hematopoietic cells to replenish, the mice were allowed to recover on a regular chow diet after the transplantation procedure. After 10 weeks recovery, chemerin was confirmed with DNA isolated from whole blood (data not shown) and the Ldlr−/− mice were then fed a HFC diet for 12 weeks to induce NASH.

Oral Glucose Tolerance Test and Insulin Tolerance Test

After 6 hours of fasting, the mice were subjected to an oral glucose tolerance test (OGTT) or an insulin tolerance test (ITT). For the OGTT, mice received a bolus of glucose (2 g/kg) and glucose was measured with a glucose meter in blood samples taken beforehand and at 15, 30, 60 and 120 minutes after gavage. For the ITT, mice were injected intraperitoneally with 0.7 U/kg insulin (WT and Cmklr1−/− mice) or 0.5 U/kg insulin (bone marrow-transplanted Ldlr−/− mice) (Actrapid, Novo Nordisk Canada Inc., Ontario, Canada) and blood glucose levels were measured at the same time points.

Blood and Tissue Collection

Mice were euthanized by a heart puncture under general anesthesia for the collection of blood and tissues. Blood was spun at 3000 g for 10 minutes at 4°C and plasma was stored at −20°C. Tissues were snap-frozen in liquid nitrogen or fixed in 4% paraformaldehyde.

Hepatic Lipid Extraction and Analysis

Lipids were extracted from crushed liver samples according to Bligh and Dyer’s method [18]. Hepatic cholesterol and triglyceride levels were determined using commercially available kits (Roche, Mannheim, Germany).

Plasma Measurements

Plasma cholesterol, triglycerides and free fatty acid levels were measured with commercially available kits (cholesterol and triglycerides: Roche; free fatty acids: DiaSys, Holzheim, Germany). Chemerin and insulin were determined in plasma from mice that had fasted for 6 hours, using commercially available ELISA kits (chemerin: R&D systems, Abingdon, UK; insulin: Alpco Diagnostics, Salem, NH).

Real-time PCR

RNA from homogenized liver, visceral and subcutaneous adipose tissue (VAT and SAT) samples was isolated according to the manufacturer’s instructions using Qiazol reagent (Qiagen, Venlo, the Netherlands). For real-time (RT)-PCR, cDNA was synthesized with a commercially available kit (Quantitect Reverse Transcription, Qiagen). RT-PCR was performed using Sybr Green Supermix (Bio-Rad, Veenendaal, the Netherlands) according to the manufacturer’s instructions. The primer sequences are listed in Table S1.

Histological Analysis

For histological analysis, paraffin-embedded liver, VAT and SAT sections (4 μm) were stained with Hematoxylin-Eosin (HE). HE-stained liver sections were scored for steatosis, lobular inflammation and hepatocyte ballooning by a board certified veterinary pathologist based on the Kleiner Scoring System [19]. The sum of these findings was used to determine the NAFLD activity score (NAS). HE-stained VAT and SAT sections were scanned using the NanoZoomer 2.0-HT slide scanner (Hannamatsu, Herrsching am Ammersee, Germany). To estimate adipocyte size, the number of adipocytes per mm2 were determined on scanned sections by manually counting all the
adipocytes in an area of 4–5 mm² using Aperio ImageScope (Leica Biosystems Imaging Inc., CA, USA). Frozen-cut liver sections (5 μm) were fixed in 4% paraformaldehyde and stained with antibodies against the macrophage markers CD68 and CD11b.

**Statistical Analysis**

For statistical analysis, non-parametric t-tests were performed using GraphPad Prism 5.0 (San Diego, USA). Data were expressed as mean ± SEM and the threshold for significance was set at p<0.05.

**Results**

**Cmklr1 Deficiency Does Not Affect Body Weight or Food Intake**

As Cmklr1 is known to be necessary for adipocyte differentiation [20] and Cmklr1⁻/⁻ mice were shown to have reduced body weight and fat mass compared to WT mice [15], we evaluated body weight gain and food intake in mice fed a HFC diet for 12 weeks. No differences in body weight or food intake were observed between WT and Cmklr1⁻/⁻ mice (Figs. 1A and 1B, respectively).
Moreover, we observed no differences in the number of adipocytes per mm² in visceral and subcutaneous adipose tissue (VAT and SAT) (VAT: WT, 143 cells/mm²; Cmklr1⁻/⁻, 155 cells/mm², SAT: WT, 301 cells/mm²; Cmklr1⁻/⁻, 345 cells/mm²), indicating that adipose tissue morphology was similar in WT and Cmklr1⁻/⁻ mice (Fig. 1C). In addition, plasma triglycerides (Fig. 1D), cholesterol (Fig. 1E) and free fatty acid levels (Fig. 1F) were the same in both genotypes. However, plasma chemerin levels were significantly increased in Cmklr1⁻/⁻ mice fed a HFC diet compared to WT mice (Fig. 1G), suggesting a compensatory upregulation of the receptor ligand. To identify the origin of these increased plasma chemerin levels, we measured chemerin expression in liver, visceral and subcutaneous adipose tissue (VAT and SAT). Chemerin expression was significantly higher in VAT and SAT, but not in the liver (Fig. S1A). As chemerin has two other receptors, G protein coupled receptor 1 (Gpr1) and (C-C motif) receptor-like 2 (Ccr12) [21,22] we also measured whether the expression of these receptors was altered. No differences were found in the expression of these receptors in either liver tissue, VAT or SAT (Figs. S1B and S1C).

Cmklr1 Deficiency Does not Affect the Development of Systemic Insulin Resistance

As the chemerin-Cmklr1 system has previously been implicated in the development of insulin resistance [15,23,24], we next assessed the glucose tolerance and insulin sensitivity in WT and Cmklr1⁻/⁻ mice fed a HFC diet for 12 weeks. However, plasma glucose (Fig. 2A) and insulin levels (Fig. 2B) did not differ between the genotypes, and the mice responded similarly to an oral bolus of glucose (Fig. 2C) and an intraperitoneal insulin injection (Fig. 2D). This indicates that Cmklr1 deficiency does not affect the glucose tolerance and insulin sensitivity of mice fed a HFC diet.

Cmklr1 Deficiency Does not Affect the Development of NAFLD

To investigate whether Cmklr1 plays a role in the development of diet-induced NAFLD, we analyzed lipid levels and gene expression in the livers of Cmklr1⁻/⁻ mice fed a HFC diet for 12 weeks. Although liver weight, expressed as a percentage of body weight, was lower in Cmklr1⁻/⁻ mice (WT, 5.70%±0.21; Cmklr1⁻/⁻, 5.16%±0.041, p<0.05), hepatic triglyceride content did not differ between WT and Cmklr1⁻/⁻ mice (Fig. 3A). In line with this, no difference in hepatic cholesterol was observed between the genotypes (Fig. 3B). The expression of genes encoding for proteins involved in macrophage activation and infiltration (Cd68 and Cd11b), inflammation (Mcp-1 and Tnfα) and fibrosis (αSma, Col1a1, Timp1 and Mmp9) was similar in Cmklr1⁻/⁻ mice and WT mice (Fig. 3C). However, Il-1β gene expression was slightly, but significantly, reduced in Cmklr1⁻/⁻ mice (Fig. 3C). Furthermore, immunostaining for CD68 and CD11b demonstrated that Cmklr1 deficiency did not affect the activation or infiltration of macrophages in the livers of mice fed a HFC diet (Fig. 3D). Pathological examination of HE-stained liver sections (Fig. 3D) confirmed these findings (Figs. S2A-C) and revealed no difference in NAS (Fig. 3E).

Hematopoietic Deletion of Cmklr1 Does not Affect the Development of Insulin Resistance or NASH

To further investigate if Cmklr1 deficiency affects the progression of insulin resistance and NAFLD, we transplanted bone marrow from Cmklr1⁻/⁻ mice or WT mice into Ldlr⁻/⁻ mice. Ldlr⁻/⁻ mice fed a HFC diet can be regarded as a model for NASH that is specifically driven by Kupffer cell activation and the recruitment of macrophages [25,26]. Ldlr⁻/⁻ mice transplanted with Cmklr1⁻/⁻ bone marrow cells (Ldlr⁻/⁻BMT⁻/⁻) had a greater gain in body weight than Ldlr⁻/⁻ mice transplanted with WT bone marrow cells.
Ldlr-BMTWT) (Fig. 4A), and this could not be explained by differences in food intake (Fig. S3A). In concordance, the number of adipocytes per mm² was lower in Ldlr-BMTCmklr1-/− mice compared to Ldlr-BMTWT mice in both VAT (Ldlr-BMTWT, 245 cells/mm²±26; Ldlr-BMTCmklr1-/−, 178 cells/mm²±15; p = 0.07) and SAT (Ldlr-BMTWT, 474 cells/mm²±51; Ldlr-BMTCmklr1-/−, 302 cells/mm²±30; p<0.05), indicating that Ldlr-BMTCmklr1-/− mice have larger adipocytes (Fig. S3B). In contrast,
we found no significant differences in plasma triglyceride, cholesterol, glucose and insulin levels between the two groups of mice (Figs. S3C-F). Moreover, hematopoietic deletion of Cmklr1 did not affect glucose tolerance (Fig. 4B) or insulin tolerance (Fig. 4C). Whereas liver weight, expressed as a percentage of body weight, was slightly lower in Ldlr-BMTWT mice compared to Ldlr-BMTCmklr1-/mice (Ldlr-BMTWT, 5.49% ± 0.086; Ldlr-BMTCmklr1-/mice, 5.08% ± 0.10, p < 0.05), the Ldlr-BMTCmklr1-/mice showed a small increase in their hepatic triglyceride content and hepatic steatosis (Fig. 4D and Fig. S3G). No differences were observed in hepatic cholesterol accumulation (Fig. 4E). As in the whole-body knock-out model, no differences in inflammatory gene expression (Cd68, Mcp-1, Tnfα, Il1-β, and Il6) were observed (Fig. 4F). Examination of pro-fibrotic genes revealed a trend towards an increase of fibrosis in Ldlr-BMTCmklr1-/mice. However, the differences were only significant for Col1a1 (Fig. 4F). Overall, these data indicate that whole-body or hematopoietic ablation of Cmklr1 does not impact on the development of systemic insulin resistance and NAFLD in mice.

Discussion

The role of Cmklr1 in the development of insulin resistance and NAFLD is controversial. Our data show that whole-body as well as hematopoietic deletion of Cmklr1 in Ldlr-/ mice did not affect the development of insulin resistance or NAFLD when the mice were fed a HFC-diet for 12 weeks. These results raise the question whether the alterations in serum chemerin levels and hepatic
Chemerin and Cmklr1 expression found in rodent models of NAFLD [7,12,14] are causally involved in the development and/or progression of this disease.

In contrast to our findings, others previously reported that Cmklr1-/− mice have reduced hepatic inflammation compared to WT mice [15], which may be related to the reduced body weight and fat mass found in these Cmklr1-/− mice. In line with this, the same investigators showed that Cmklr1-/− mice have reduced steatosis on a low fat diet. However, these differences were not present on a high fat diet [15]. Our results confirm their latter findings. Consistent with our results, another paper observed no differences in body weight, fat mass and glucose tolerance between WT and Cmklr1-/− mice at a young age. In that study, Cmklr1-/− mice started to gain more fat mass than WT mice only from age 8 months onwards [16]. The increased body weight gain (Fig. 4A) and adipocyte size (Fig. S3B) found in Ldlr-/− mice with a hematopoietic deletion of Cmklr1 might therefore be an age-induced effect. These mice were euthanized at 7–10 months of age, whereas the whole-body knock-outs were euthanized at 6–7 months of age. These results suggest that hematopoietic cells might be responsible for the age-induced weight gain of Cmklr1-/− mice. In contrast to Cmklr1−/− mice, the Ldlr-BMT-Cmklr1−/− mice had increased hepatic triglyceride levels (Fig. 4D), increased hepatic steatosis (Fig. S3G) and a tendency towards an increased expression of pro-fibrotic genes (Fig 4F). However, these differences were small and may well be explained by the increased body weight gain seen in these mice.

As neither whole-body Cmklr1 deficiency and hematopoietic deletion of Cmklr1 in Ldlr−/− mice affected the development of NAFLD, the alterations in chemerin and Cmklr1 levels found in humans and mouse models of NAFLD may be a secondary effect of the metabolic syndrome and NAFLD [10–12,14]. In these disorders, many of the factors that can regulate chemerin and Cmklr1 expression become dysregulated. First, chemerin is an adipokine and its secretion rises with increasing adiposity. Second, increased chemokine-like receptor 1 knock-out. N = 8 for all experiments. Data are expressed as mean ± SEM.

Supporting Information

Figure S1 The expression of chemerin is increased in adipose tissue, but not in the liver. The expression of chemerin (A) and its receptors, Gpr1 (B) and Ccr2 (C), was measured in liver, visceral and subcutaneous adipose tissue of WT mice (white bars) and Cmklr1−/− mice (black bars) fed a high fat, high cholesterol diet for 12 weeks. Abbreviations: WT, wild type; Cmklr1−/−, chemokine-like receptor 1 knock-out; VAT, visceral adipose tissue; SAT, subcutaneous adipose tissue; Gpr1, G protein-coupled receptor 1; Ccr2, (C-C) motif receptor-like 2. N = 6–8 for all experiments. Data are expressed as mean ± SEM.

Figure S2 Steatosis, lobular inflammation and ballooning scores were not affected by Cmklr1 deficiency. Hematoxylin-Eosin (HE) stained liver sections of mice fed a high fat, high cholesterol diet for 12 weeks were scored for steatosis (A), lobular inflammation (B) and ballooning (C) by a certified veterinary pathologist. Abbreviations: WT, wild type; Cmklr1−/−, chemokine-like receptor 1 knock-out. N = 8 for all experiments. Data are expressed as mean ± SEM.

Figure S3 Characteristics of bone marrow transplanted Ldlr−/− mice. (A) Food intake was measured throughout the 12-week high fat, high cholesterol (HFC) diet period and calculated in grams per day. (B) Representative pictures were taken of Hematoxylin-Eosin (HE) stained liver sections of mice fed a high fat, high cholesterol diet for 12 weeks were scored for steatosis (A), lobular inflammation (B) and ballooning (C) by a certified veterinary pathologist. Abbreviations: WT, wild type; Cmklr1−/−, chemokine-like receptor 1 knock-out. N = 5–7 for all experiments. Data are expressed as mean ± SEM.

Table S1 Primer sequences.

Acknowledgments

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**Author Contributions**

Conceived and designed the experiments: NG MAV DPYK. Performed the experiments: NG MAV NJK HVDM SS SY. Analyzed the data: NG MAV NJK HVDM SS SY AB DJD JAK BVDS MHH DPYK.

**References**