Genetic inactivation of the LIGHT (TNFSF14) cytokine in mice restores glucose homeostasis and diminishes hepatic steatosis

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Abstract

Aims/hypothesis Non-alcoholic fatty liver disease (NAFLD) is frequently associated with type 2 diabetes mellitus. Progression of NAFLD is mediated, among other things, by activation of inflammatory pathways. In the present study, the role of the proinflammatory cytokine LIGHT (TNFSF14) was explored in NAFLD and type 2 diabetes mellitus in mice deficient for the cytokine.

Methods *Light*-deficient (*Light*^{−/−}) mice and WT controls were fed a regular chow diet (RCD) or a high-fat high-cholesterol diet (HFHCD) for 16 weeks. The expression of LIGHT and its receptors, herpes virus entry mediator (HVEM) and lymphotoxin β receptor (LTβR), was investigated in both dietary regimens. Glucose tolerance, insulin sensitivity, non-alcoholic fatty liver (NAFL), systemic and tissue inflammation, and metabolic gene expression were explored in *Light*^{−/−} and WT mice fed an RCD and an HFHCD. The effect of *Light* deficiency was also evaluated in hepatic tissue and in inflammation in HFHCD-fed *Irs2*^{+/-} mice with impaired insulin signalling.

Results *Light* deficiency did not have an effect on metabolism, in NAFL or in tissue and systemic inflammation, in RCD-fed WT mice. HVEM and LTβR were markedly increased in livers of HFHCD-fed WT mice compared with RCD-fed WT controls. In WT mice under HFHCD, *Light* deficiency improved glucose tolerance and insulin sensitivity. Non-alcoholic fatty liver disease activity (NAS) score, hepatic CD3⁺ T lymphocytes and F4/80⁺ macrophages were decreased in HFHCD-fed *Light*^{−/−} mice compared with HFHCD-fed WT controls. Consistent with a potential role of adipose tissue in hepatic homeostasis, *Light*^{−/−} mice exhibited augmented anti-inflammatory F4/80⁺CD206⁺ adipose tissue macrophages and reduced proinflammatory F4/80⁺CD11c⁺ adipose tissue macrophages. Moreover, adipose tissue explants from *Light*^{−/−} mice showed diminished secretion of monocyte chemoattractant protein 1 (MCP1), TNF-α and IL-17 cytokines. Circulating *Light*^{−/−} leucocytes consistently displayed augmented levels of the patrolling Ly6C^{low} monocytes, decreased Th9 T cell subset and diminished plasma TNF-α and IL-6 levels. Similarly, *Light* deficiency in *Irs2*^{+/-} mice, which display impaired insulin signalling, also reduced NAFL as well as systemic and adipose tissue inflammation. Analysis of hepatic gene expression in *Light*^{−/−} mouse livers showed reduced levels of *Zbtb16*, the transcription factor essential for natural killer T (NKT) cell function, and two genes related to NAFLD and fibrosis, *Klf6* and *Tlr4*.

Conclusions/interpretation These results indicate that *Light* deficiency in HFHCD improves hepatic glucose tolerance, and reduces hepatic inflammation and NAFL. This is accompanied by decreased systemic inflammation and adipose tissue cytokine secretion and by changes in the expression of key genes such as *Klf6* and *Tlr4* involved in NAFLD. These results suggest that therapies to block LIGHT-dependent signalling might be useful to restore hepatic homeostasis and to restrain NAFLD.

Keywords Adipose tissue · Glucose intolerance · Hepatic steatosis · Insulin resistance · LIGHT (TNFSF14) · Metabolism

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Research in context

What is already known about this subject?

- Hepatic inflammation promotes the development of non-alcoholic fatty liver disease (NAFLD)
- LIGHT (TNFSF14) is a proinflammatory cytokine increased in individuals with NAFLD and type 2 diabetes mellitus
- Cholesterol-rich diets and insulin resistance are both factors that predispose to NAFL-to-NASH (non-alcoholic steatohepatitis) transition

What is the key question?

- Does LIGHT modulate carbohydrate metabolism and progression of NAFLD in a cholesterol-rich diet?

What are the new findings?

- A high-fat high-cholesterol diet (HFHCD) in WT mice promotes hepatic LIGHT/LT β R-HVEM-mediated signalling by increasing receptor expression
- *Light* deficiency in HFHCD-fed mice improves glucose tolerance, insulin sensitivity and NAFL, and reduces hepatic, adipose tissue and systemic inflammation
- *Light* deficiency decreases hepatic expression of genes associated with the NAFL-to-NASH transition, such as *Klf6* and *Tlr4*

How might this impact on clinical practice in the foreseeable future?

- Modulation of LIGHT-dependent signalling might be a therapeutic avenue to reduce the transition of NAFL to NASH associated with insulin resistance

Abbreviations

ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ATM	Adipose tissue macrophage
CCR	C-C chemokine receptor
CXCR	CXC chemokine receptor
HCC	Hepatocellular carcinoma
HFHCD	High-fat high-cholesterol diet
HVEM	Herpes virus entry mediator
LIGHT	Homologous to Lymphotoxin, exhibits Inducible expression and competes with HSV Glycoprotein D for binding to Herpesvirus entry mediator, a receptor expressed on T lymphocytes
LT β R	Lymphotoxin β receptor
MCP1	Monocyte chemoattractant protein 1
NAFL	Non-alcoholic fatty liver
NAFLD	Non-alcoholic fatty liver disease
NAS	Non-alcoholic fatty liver disease activity (score)
NASH	Non-alcoholic steatohepatitis
NKT	Natural killer T
RCD	Regular chow diet
Th	T helper
TNFSF14	TNF superfamily member 14
SVF	Stromal vascular fraction
WT	Wild-type

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a major metabolic complication frequently associated with insulin resistance, a key feature of type 2 diabetes mellitus. Both of these metabolic alterations are becoming health problems due to the increasing obesity and sedentary lifestyle patterns in the population, which predispose to these pathologies [1].

Remarkably, NAFLD develops in up to 90% of obese and up to 70% of overweight people [2]. The excess of fat in adipose tissue and adipocyte hypertrophy activate NEFA release which accumulates in the liver, promoting non-alcoholic fatty liver (NAFL) [3]. Moreover, sustained adipose tissue expansion might activate stress-induced mitogen-activated protein kinases, generating adipose tissue dysfunction and promoting the release of proinflammatory cytokines into the bloodstream which facilitate inflammation-induced insulin resistance and NAFL. While NAFL is asymptomatic per se, hepatic inflammation induces the transition to non-alcoholic steatohepatitis (NASH), a hepatic dysfunctional metabolic state. Interestingly, some studies in humans indicate that NASH might even develop before steatosis in some cases such as hepatic cholesterol accumulation [4], which induces inflammation and hepatic uptake of fatty acids. Current hypotheses on the NAFL-to-NASH transition involve many

factors such as defective insulin signalling, characteristic of insulin resistance, genetic predisposition and environmental factors such as cholesterol-enriched diets [5, 6].

LIGHT (TNFSF14) (Homologous to Lymphotoxin, exhibits Inducible expression and competes with HSV Glycoprotein D for binding to Herpesvirus entry mediator, a receptor expressed on T lymphocytes [TNF superfamily member 14]) is a soluble inflammatory cytokine that belongs to the TNF superfamily of proinflammatory molecules [7]. LIGHT is mainly produced by T cells and monocytes, and mediates its effects through three receptors, the lymphotoxin β receptor (LT β -R), the herpes virus entry mediator (HVEM) and decoy receptor 3 (DcR3) which inhibits its function [8]. Previous studies have shown that LIGHT-deficient macrophages display reduced production of cytokines [7], while constitutive overexpression of LIGHT promotes T lymphocyte activation and maturation and leads to severe inflammation and tissue destruction [9]. A role for LIGHT has also been suggested in hepatic homeostasis since investigations in humans associate enhanced levels of the cytokine with NAFLD [10], with insulin resistance and the metabolic syndrome [11], and with type 2 diabetes mellitus [12]. These studies suggest a function of LIGHT in the development of liver dysfunction-related diseases.

In the present study, we investigated whether genetic inactivation of LIGHT modulates hepatic metabolic diseases, insulin resistance and NAFLD, and potential underlying molecular mechanisms. To this end, metabolism, inflammation and hepatic derangement were investigated in *Light*^{-/-} and wild-type (WT) mice fed a regular chow diet (RCD) and a high-fat high-cholesterol diet (HFHCD) for 16 weeks (NB *Light* is also known as *Tnfsf14*). In addition, studies were also performed in *Irs2*^{+/-} mice, which display impaired insulin signalling [13].

Methods

Generation of mice, diets and treatments The experimental (*Light*^{-/-} *n* = 46 and *Irs2*^{+/-}*Light*^{-/-} *n* = 21) and control groups (WT = 36 and *Irs2*^{+/-} *n* = 26) were generated by crosses between WT, *Light*^{-/-} and *Irs2*^{+/-} mice at INCLIVA animal facility (all in C57BL/6J background). Mice had free access to water, and were under temperature- and humidity-controlled conditions (22 ± 1°C, 40–45%) with 12 h light/dark cycles in a conventional animal facility. Mice were maintained on an RCD (2.8% fat; Panlab, Barcelona, Spain) and at 8 weeks of age were randomly placed on an HFHCD (10.8% fat, 0.75% cholesterol; S4892-E010, Ssniff, Soest, Germany) or left on an RCD for 16 more weeks. Analysis was performed in male and female mice. Animal procedures were approved by the Animal Ethics Committee of INCLIVA and followed the 2010/63/EU directive from the European Parliament.

Plasma measurements, body and liver weight and fat mass distribution, and metabolic studies Biochemical determinations were performed in isolated plasma from whole blood in the presence of EDTA from overnight-fasted mice. Analysis included triacylglycerol, total cholesterol, ApoB-cholesterol, HDL-cholesterol [14], alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities following the indicated protocols. GTTs and ITTs [15] were performed in overnight- and 4 h-fasted mice, respectively, and glucose and insulin levels were measured as reported [16]. For liver weight and body fat mass distribution, liver and inguinal, epididymal and mesenteric fat pads were dissected from mouse bodies, weighed and expressed as percentage of total body weight. See [electronic supplementary material](#) (ESM) Methods.

Hepatic triacylglycerol content Triacylglycerol hepatic content was determined by tissue digestion and saponification in ethanolic potassium hydroxide [17], followed by an enzymatic measurement of glycerol content (Free Glycerol Reagent, Sigma, St Louis, MO, USA).

Western blot analysis Protein extracts were obtained by homogenising snap-frozen liver and adipose tissue as described [18] and prepared for immunoblot analysis using specific primary antibodies, anti-LT β R, anti-HVEM and anti- β -actin, followed by secondary horseradish peroxidase (HRP)-conjugated antibodies and detected by chemiluminescence. See [electronic supplementary material](#) (ESM) Methods.

Immunohistopathological characterisation of liver Livers were sectioned from mice, washed with PBS, fixed with 4% paraformaldehyde/PBS and paraffin embedded [13, 18]. Hepatic macrophage content was determined by anti-F4/80 marker immunohistochemistry [13]. For CD3, F4/80-LT β R, F4/80-HVEM and F4/80-LIGHT immunofluorescences, cross-sections were treated for antigen retrieval and incubated with primary antibodies followed by fluorescent secondary antibody [15]. Liver complications were assessed by steatosis, hepatocellular ballooning, lobular inflammation and non-alcoholic fatty liver disease activity (NAS) score in haematoxylin–eosin-stained sections and fibrosis in Masson trichrome-stained sections [19]. See [electronic supplementary material](#) (ESM) Methods.

Flow cytometry analysis of stromal vascular fraction cells from adipose tissue Stromal vascular fraction (SVF) cells were isolated by digestion of epididymal fat pads with 1 mg/ml type I collagenase (Worthington, Lakewood, NJ, USA) at 37°C for 30 min. SVF cells were washed, fixed with Zombie Aqua fixable viability kit (BioLegend, San Diego, CA, USA) and stained (30 min at room temperature) with anti-mouse F4/80-APC (MCA497APTC, Bio-Rad, Hercules,

CA, USA), anti-mouse CD11c-PE (12-0114-81, eBioscience, San Diego, CA, USA) and anti-mouse CD206-AlexaFluor488 (MCA2235A488T, Bio-Rad). Stains were followed by incubation with FACS lysing solution for 15–20 min and flow cytometry analysis using FACSVerse (BD Biosciences, San Jose, CA, USA) to determine proinflammatory M1 F4/80⁺CD11c⁺CD206⁻ adipose tissue macrophages (ATMs) and alternative M2 F4/80⁺CD11c⁻CD206⁺ ATMs.

ELISA Circulating levels of cytokines were determined in isolated plasma from heparinised mouse blood (10 U heparin/ml) obtained from different mice using the specific cytokine DuoSet ELISA kits (R&D Systems, Minneapolis, MN, USA).

Circulating leucocyte analysis by flow cytometry To characterise circulating leucocytes, 10 µl heparinised whole blood was incubated for 30 min at room temperature with CD45-FITC, Ly6C-PerCP (553080 and 560525, BD Pharmingen, Franklin Lakes, NJ, USA) and CD115-APC (135509, BioLegend) for monocyte analysis. For identification of T helper (Th) cell subsets in mice, the human protocol was adapted for mouse blood as described [20, 21]. Mouse blood was also incubated (30 min, room temperature) with BV421-C-C chemokine receptor (CCR)4 (CD194) (131217, BioLegend), CD4-FITC (130-102-541, Miltenyi, Bergisch Gladbach, Germany), CXCR chemokine receptor (CXCR)3 (CD183)-APC (17-1831-80, eBioscience) and CCR6(CD196)-PEVio770 (130-103-818, Miltenyi). Stains were followed by incubation with FACS lysing solution (BD Biosciences) for 15–20 min before flow cytometry analysis using FACSVerse or Fortessa Flow cytometers (BD Biosciences).

Adipose tissue explant isolation Epididymal fat pads were minced into 2–3 mm³ and incubated for 24 h at 100 mg/ml in six-well plates with DMEM-P/S/A-10%FBS medium in a humidified 5% CO₂ atmosphere. Explants were washed with PBS1X twice and incubated with DMEM-P/S/A-0.1%FBS medium for 24 h, and the medium was collected for cytokine analysis by ELISA.

Experiments with bone marrow-derived macrophages Cells from femoral bone marrow were cultured at 1×10^6 cells/ml density and differentiated over 7 days [15], then incubated for 24 h with serum-free medium for cytokine secretion analysis. See [electronic supplementary material](#) (ESM) Methods.

Gene expression analysis by quantitative real-time PCR RNA (0.5–1 µg) from mouse tissues was obtained using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), reverse-transcribed with the Maxima First-Strand kit and amplified with Luminaris Color qPCR MasterMIX (Fermentas,

Waltham, MA, USA) on a 7900 FastSystem thermal cycler. Results were analysed with the provided software (Applied Biosystems, Foster city, CA, USA). mRNA levels were normalised to the cyclophilin expression and relativised to controls. The primer sequences can be found in ESM Table 1.

Statistical analysis Quantitative variables are shown as single data points and mean $\pm$ SEM. Mouse sample replicates for in vivo data were obtained from individual mice. For adipose tissue and macrophage experiments, replicates were obtained from 4–6 samples derived from three or two independent mice per genotype. All data obtained were analysed by observers blinded to genotype and the criteria for exclusion were data out of range of the standard curve or samples that were lost during the experiment. Data were analysed for normal distribution using D'Agostino–Pearson omnibus test. Differences were evaluated with Student's *t* test (two groups, paired test), Mann–Whitney *U* test (nonparametric test), one-way ANOVA followed by Bonferroni multiple comparison test (more than two mouse groups) and two-way ANOVA followed by Tukey's post hoc multiple comparison test (two mouse groups and two treatments) (GraphPad Prism Software, La Jolla, CA, USA). Statistical significance was set at $p \leq 0.05$. Male and female data were displayed together, and when sex-dependent responses were observed data were presented separately.

Results

HFHCD increases LTβR and HVEM expression in WT mice To evaluate the impact of HFHCD on LIGHT-mediated signalling in WT mice, circulating LIGHT levels and expression of LTβR and HVEM receptors in liver and adipose tissue were explored. Circulating LIGHT levels were similar between RCD- and HFHCD-fed WT mice (Fig. 1a). mRNA expression analysis showed enhanced levels of *Light* in liver in HFHCD-fed WT mice (Fig. 1b) but no changes in adipose tissue gene expression (Fig. 1c). Protein expression analysis of LIGHT receptors revealed augmented hepatic and adipose tissue levels of LTβR and HVEM (Fig. 1d–i) in HFHCD-fed WT mice compared with RCD-fed WT controls. Double immunofluorescences of LIGHT, LTβR and HVEM with F4/80 macrophage marker showed most of the receptor expression in parenchymal hepatic cells (Fig. 1j, k), while LIGHT levels were low and localised in macrophages as well as in other cell types (Fig. 1l). These data indicate that HFHCD feeding induces an increase in the LIGHT/LTβR-HVEM-dependent signalling.

Light deficiency improves glucose tolerance, insulin sensitivity and NAFLD in WT mice fed HFHCD Analysis of mice fed an RCD did not show differences in body weight, fat mass content or distribution between *Light*^{-/-} and WT mice or in liver

Fig. 1 LIGHT/LT β R-HVEM expression analysis in liver and adipose tissue from WT mice fed an RCD and an HFHCD. **(a)** Circulating plasma levels of LIGHT in mice fed an RCD and an HFHCD. **(b)** mRNA levels of *Light* in (b) liver and (c) adipose tissue. Protein levels of **(d, g)** LT β R and **(e, h)** HVEM in liver **(d–f)** and adipose tissue **(g–i)** homogenates in RCD-fed and HFHCD-fed WT mice. **(f, i)** Representative blots showing LT β R and HVEM levels in **(f)** liver and **(i)** adipose tissue. Double immunofluorescence of **(j)** F4/80-LT β R, **(k)** F4/80-HVEM and **(l)** F4/80-LIGHT in WT mice fed an RCD and an HFHCD. Triangles, RCD-fed mice; circles, HFHCD-fed mice; white, male WT mice; grey, female WT mice. Scale bars, 50 μ m, and 20 μ m in the magnifications. mRNA levels were normalised to endogenous cyclophilin expression and are relative to RCD-fed mouse levels. Total protein levels were normalised to β -actin. Data are shown as individual data points with mean $\pm$ SEM. Statistical analysis was performed using Student's *t* test (**p* < 0.05, ***p* < 0.01 and ****p* < 0.001). AT, adipose tissue

weight (ESM Fig. 1a–d). Similarly, fasting glucose and insulin levels (Fig. 2a, b) were indistinguishable between mouse groups. No changes were observed in the GTT measured as the AUC_{glucose} variable, or in glucose-stimulated insulin release, measured as AUC_{insulin}, or in the ITT (Fig. 2c–e). Similarly, *Light* deficiency did not change body weight, body fat mass content or distribution, liver weight (ESM Fig. 2a–d), fasting glucose or insulin levels (Fig. 2f, g) in mice placed on

HFHCD. However, GTT revealed improved glucose tolerance, measured as the AUC_{glucose} variable (Fig. 2h), in *Light*-deficient mice compared with controls. No differences were observed in the glucose-stimulated insulin release during the GTT, measured as the AUC_{insulin} at 120 min of the test (Fig. 2i). However, AUC_{insulin} at 30 min in *Light*^{−/−} mice displayed an increase in insulin release compared with WT mice (Fig. 2i). ITT demonstrated improved insulin sensitivity in *Light*^{−/−}

Fig. 2 Metabolic characterisation of WT and *Light*^{-/-} mice fed for 16 weeks with an RCD or an HFHCD. Plasma fasting (a) glucose and (b) insulin levels in WT and *Light*^{-/-} mice fed an RCD. Plasma (c) glucose and (d) insulin levels at the different time points during the GTT, which were used to calculate the AUC_{glucose} (e) and AUC_{insulin} (f), in RCD-fed WT and *Light*^{-/-} mice. (e) Plasma glucose levels (as a percentage relative to the initial glucose levels) during the ITT, together with the AUC_{glucose}, in RCD-fed WT and *Light*^{-/-} mice. Plasma fasting (f) glucose and (g) insulin levels in WT and *Light*^{-/-} mice fed HFHCD. Plasma fasting (h) glucose and (i) insulin levels at the different time points

during the GTT and the AUC_{glucose} (h) and AUC_{insulin} at 120 and 30 min (i) in WT and *Light*^{-/-} mice fed HFHCD. (j) Plasma glucose levels (as a percentage relative to the initial glucose levels) during the ITT, together with AUC_{glucose}, in mice fed HFHCD. Triangles, RCD-fed mice; circles, HFHCD-fed mice; white, male WT mice; grey, female WT mice; red, male *Light*^{-/-} mice; blue, female *Light*^{-/-} mice; hatched lines indicate both male and female mice. The line graphs in (c–e) and (h–j) show the mean of males and females. All other data are shown as individual data points with mean $\pm$ SEM. Statistical analysis was performed using Student's *t* test (**p* < 0.05 and ****p* < 0.001)

mice, as revealed by a decrease in the AUC_{glucose} (Fig. 2j). Analysis of lipids indicated no effect of *Light* deficiency in total cholesterol, ApoB-cholesterol, HDL-cholesterol and triacylglycerol either in mice fed an RCD (ESM Fig. 1e–h) or mice fed HFHCD (ESM Fig. 2e–h). Therefore, *Light* deficiency does not have an effect in mice fed an RCD, but improves glucose tolerance and insulin sensitivity in mice fed HFHCD without changes in lipid plasma levels.

Light deficiency in RCD-fed mice did not affect hepatic triacylglycerol content, steatosis, ballooning, lobular inflammation or NAS score (Fig. 3a, c, e, g, i). But, *Light*^{-/-} mice fed HFHCD showed significantly diminished hepatic triacylglycerol content (Fig. 3b), hepatic steatosis (Fig. 3d), lobular inflammation (Fig. 3h) and NAS score (Fig. 3j) without changes in ballooning score (Fig. 3f). No differences in hepatic F4/80⁺ macrophage and CD3⁺ lymphocyte contents or collagen in

Fig. 3 Hepatic characterisation in WT and *Light*^{-/-} mice fed for 16 weeks with an RCD and HFHCD. Analysis of (a, b) triacylglycerol content, (c, d) steatosis score, (e, f) hepatocyte ballooning, (g, h) lobular inflammation and (i, j) NAS score, in mice fed an RCD (a, c, e, g, i) and HFHCD (b, d, f, h, j). (k) Representative images of steatosis, hepatocyte ballooning and lobular inflammation. Scale bars, 50 μ m. Triangles, RCD-fed mice; circles, HFHCD-fed mice; white, male WT mice; grey, female WT mice; red, male *Light*^{-/-} mice; blue, female *Light*^{-/-} mice. Data are shown as individual data points with mean $\pm$ SEM. Statistical analysis was performed using Student's *t* test (a, b, d, i, j) and Mann–Whitney *U* test (c, e–h) (**p* < 0.05, ***p* < 0.01 and ****p* < 0.001)

RCD-fed mice (Fig. 4a, d, g) were observed, but *Light*^{-/-} mice fed HFHCD displayed diminished macrophage and CD3⁺ lymphocytes compared with controls (Fig. 4b, e) without changes in fibrosis (Fig. 4h). No changes were seen in the activity of ALT or AST between genotypes in any dietary regimen (Fig. 4j, k, l, m). Comparison between the two dietary regimens in WT mice showed enhanced triacylglycerol and F4/80⁺ macrophage contents (ESM Fig. 3a, b) in mice fed HFHCD.

Analysis of hepatic gene expression showed augmented mRNA levels of *Cd11c*, *Zbtb16* and *Tbet* (Fig. 5a, c, d), which are determinant for proinflammatory macrophages, natural killer T (NKT) cell identity and proinflammatory Th1, respectively, in HFHCD-fed WT mice compared with RCD-fed WT controls. Consistently, mRNA expression of *Cd206*, *Gata3* and *Foxp3* (Fig. 5b, e, g), markers of anti-inflammatory macrophages, Th2 subset and Treg cells, respectively, were significantly decreased in WT and *Light*^{-/-} HFHCD-fed mice compared with their respective RCD-fed mice. Comparison between *Light*^{-/-} and WT mice indicated decreased mRNA expression of *Zbtb16* in mice fed HFHCD (Fig. 5c) and diminished anti-inflammatory *Cd206* and *Foxp3* (Fig. 5b, g) in *Light*^{-/-} mice compared with controls fed an RCD. mRNA

expression analysis of genes related to hepatic stellate cell activation, *Col1a1* and *Acta2*, or genes associated with fibrosis, such as cytokine *Tgfb1*, *Mmp9* and *Timp1*, did not display changes among mouse groups (Fig. 5h–l).

These data indicate that HFHCD induces inflammation which is alleviated by *Light* deficiency.

Deficiency of *Light* reduces adipose tissue and systemic inflammation in WT mice fed HFHCD NAFLD and hepatic inflammation might be induced by dysfunctional visceral adipose tissue [22]. Analysis of male mouse adipose tissue showed decreased cytokine secretion of monocyte chemoattractant protein 1 (MCP1), TNF- α and IL-17 by adipose tissue explants from *Light*^{-/-} mice compared with those secreted by WT controls (Fig. 6a, b, d). No differences were observed in IL-6 secretion from adipose tissue between mouse groups (Fig. 6c). In agreement with reduced proinflammatory cytokine secretion in adipose tissue, analysis of SVFs revealed a reduced percentage of the proinflammatory F4/80⁺CD11c⁺ ATMs in female mice (Fig. 6i) and augmented levels of the anti-inflammatory F4/80⁺CD206⁺ ATMs in *Light*^{-/-} male mice (Fig. 6g) without changes in total F4/80⁺ ATMs (Fig. 6e, h). Analysis of the transcription factor determinants of immune cellular subset

Fig. 4 Hepatic characterisation in WT and *Light*^{-/-} mice fed for 16 weeks with an RCD and an HFHCD. Hepatic cross-sectional analysis of (a–c) macrophage F4/80⁺ and (d–f) lymphocyte CD3⁺ contents (cell number relative to hepatic area in μm^2) in WT and *Light*^{-/-} mice fed an RCD (a, d) or HFHCD (b, e). (c, f) Representative images of (c) F4/80 immunohistochemistry and (f) CD3 immunofluorescence for both dietary regimens and genotypes. (g–i) Percentage of collagen in Masson trichrome staining in WT and *Light*^{-/-} mice fed (g) an RCD or (h) an HFHCD. Representative images of (i) collagen stainings are shown for both dietary regimens and genotypes. Plasma levels of (j, k) ALT and (l, m) AST activity in RCD-fed (j, l) and HFHCD-fed (k, m) mice. Scale bars, (c, i) 50 μm and (f) 20 μm . Triangles, RCD-fed mice; circles, HFHCD-fed mice; white, male WT mice; grey, female WT mice; red, male *Light*^{-/-} mice; blue, female *Light*^{-/-} mice. Data are shown as individual data points with mean $\pm$ SEM. Statistical analysis was performed using Student's *t* test (a, b, d, g, h, j–m) and Mann–Whitney *U* test (c) ([†]*p* = 0.05 and **p* < 0.05)

differentiation *Tbet*, *Gata3*, *Rorc* and *Foxp3* did not reveal differences between *Light*^{-/-} and WT mice (Fig. 6l–o).

Surprisingly, in contrast with these results, analysis of bone marrow-derived macrophages from *Light*^{-/-} female mice showed enhanced TNF- α and IL-6 secretion and diminished IL-10 levels compared with WT female controls (ESM Fig. 4b–d), thus indicating an intrinsic proinflammatory phenotype of *Light*-deficient macrophages. However, in vivo analysis showed diminished TNF- α and IL-6 circulating levels (Fig. 7b, c). Likewise, in vivo *Light* deficiency reduced total CD45⁺CD115⁺ monocytes in male mice (Fig. 7g), enhanced anti-inflammatory CD45⁺CD115⁺Ly6C^{low} monocytes in both sexes (Fig. 7g, i) and decreased proinflammatory CD45⁺CD115⁺Ly6C^{hi} monocytes in female mice (Fig. 7i). In addition, the CD4⁺CCR4⁻CCR6⁺ Th9 lymphocyte subset (Fig. 7n) was also reduced in HFHCD-fed *Light*^{-/-} mice

compared with WT controls. No major effect of *Light* deficiency was observed in circulating leucocytes (ESM Fig. 5a–h) or in the adipose tissue macrophage phenotype of adipose tissue (ESM Fig. 5j–l) in RCD-fed mice. Of note, the CCR4⁺CCR6⁺ Th9 population was increased in *Light*^{-/-} mice (ESM Fig. 5g). Hence, *Light* deficiency alleviates in vivo systemic and adipose tissue inflammation in HFHCD-fed mice.

Deficiency of *Light* reduces NAFLD, adipose tissue and systemic inflammation in *Irs2*^{+/-} mice fed HFHCD Next, *Light* deficiency was evaluated in the hepatic physiopathology in *Irs2*^{+/-} mice, which display enhanced insulin resistance and NAFL during aging compared with age-matched WT [13]. *Light* deficiency diminished triacylglycerol (ESM Fig. 6a) and F4/80⁺ macrophage (ESM Fig. 6b) contents in *Irs2*^{+/-} mice. As expected, compared with WT mice, *Irs2*^{+/-} mice displayed

Fig. 5 Hepatic inflammatory and fibrosis marker analysis in WT and *Light*^{-/-} mice fed with an RCD and an HFHCD. mRNA levels of (a) *Cd11c* and (b) *Cd206* macrophage surface markers, and (c) *Zbtb16*, (d) *Tbet*, (e) *Gata3*, (f) *Rorc* and (g) *Foxp3* transcription factors in liver from all four groups of mice. mRNA levels of (h) *Col1a1*, (i) *Acta2*, (j) *Tgfb1*, (k) *Mmp9* and (l) *Timp1* in liver from all four groups of mice. mRNA levels were normalised to cyclophilin mRNA levels and are relative to

levels in RCD-fed WT mouse. Triangles, RCD-fed mice; circles, HFHCD-fed mice; white, male WT mice; grey, female WT mice; red, male *Light*^{-/-} mice; blue, female *Light*^{-/-} mice. Data are shown as individual data points with mean $\pm$ SEM. Statistical analysis was performed using two-way ANOVA followed by Tukey's post hoc multiple comparison test (* p < 0.05, ** p < 0.01 and *** p < 0.001)

enhanced hepatic triacylglycerol and macrophage contents regardless of the presence of LIGHT (ESM Fig. 6d, e), indicating enhanced NAFLD in *Irs2*^{+/-} mice fed HFHCD. Circulating TNF- α and IL-17 cytokines (ESM Fig. 7b, d) were decreased in *Irs2*^{+/-} *Light*^{-/-} mice compared with *Irs2*^{+/-} mice without differences in the other cytokines analysed (ESM Fig. 7a, c, e). Circulating leucocyte analysis showed no changes in lymphocytes, monocytes or neutrophils (ESM Fig. 7f) between mouse groups. *Irs2*^{+/-} *Light*^{-/-} mice displayed augmented Ly6C^{low} monocyte percentage and CD4⁺CCR4⁺CCR6⁻ Th2 cells (ESM Fig. 7g, i), and diminished CD4⁺CXCR3⁺ Th1, CD4⁺CCR4⁺CCR6⁺ Th9 and CD4⁺CCR4⁺CCR6⁺ Th17 subsets (ESM Fig. 7h, j, k). *Irs2*^{+/-} *Light*^{-/-} mouse adipose tissue also displayed diminished secretion of TNF- α and IL-17 cytokines (ESM Fig. 7m, o) and diminished proinflammatory F4/80⁺CD11c⁺ ATMs and increased anti-inflammatory F4/80⁺CD206⁺ ATMs (ESM Fig. 7q, r). No changes in MCP1 and IL-6 secretion (ESM Fig. 7l, n) or in total F4/80⁺ ATMs (ESM Fig. 7p) were observed. Thus, *Light* deficiency ameliorates NAFL and associated inflammation in *Irs2*^{+/-} mice fed HFHCD.

Genetic inactivation of *Light* modulates hepatic expression in WT mice fed HFHCD LIGHT-dependent signalling might modulate metabolic gene expression in the liver [23]; thus, expression studies of hepatic metabolic genes related to lipogenesis, β -oxidation and gluconeogenesis were performed. Analysis showed decreased levels of *Lxr*, *Pparg*, *Ppara* and *Abca1* in *Light*^{-/-} mice (Fig. 8a, d, f, m), while *Srebf1c*, *Fasn* and *Ldlr* mRNA levels were enhanced (Fig. 8b, e, l). Expression analysis of the Krüppel-like factor (KLF) family genes showed reduced mRNA levels of *Klf6* in *Light*^{-/-} mice compared with WT controls (Fig. 8s). Similarly, *Light*^{-/-} mice displayed diminished *Tlr4* levels (Fig. 8u). Thus, *Light* deficiency might reduce NAFLD by diminishing *Klf6* and *Tlr4*, which associate with NASH.

Discussion

NAFLD is a major metabolic complication associated with type 2 diabetes mellitus and adipose tissue dysfunction. In the present study, we showed that genetic deficiency of

Fig. 6 Adipose tissue characterisation in WT and *Light*^{-/-} mice fed with HFHCD. (a) MCP1, (b) TNF- α , (c) IL-6 and (d) IL-17 cytokine secretion levels in adipose tissue from WT and *Light*^{-/-} mice. Analysis by flow cytometry of (e, h) total F4/80⁺, (f, i) proinflammatory F4/80⁺CD11c⁺ and (g, j) anti-inflammatory F4/80⁺CD206⁺ macrophage content in live cells of SVFs from adipose tissue in males (e–g) and females (h–j). (k) Representative flow cytometry plots displaying the gating strategy for the different populations. mRNA levels of (l) *Tbet*, (m) *Gata3*, (n) *Rorc* and (o) *Foxp3* transcription factors in adipose tissue from both groups of mice. mRNA levels were normalised to cyclophilin mRNA levels and are relative to levels in control WT mouse. Circles, HFHCD-fed mice; white, male WT mice; grey, female WT mice; red, male *Light*^{-/-} mice; blue, female *Light*^{-/-} mice. Data are shown as individual data points with mean $\pm$ SEM. Statistical analysis was performed using Student's *t* test (**p* < 0.05 and ****p* < 0.001). FSC, forward scatter

Light, a proinflammatory cytokine, did not affect metabolism, NAFL or inflammation in WT mice fed an RCD. However, *Light*^{-/-} mice fed HFHCD displayed improved glucose tolerance, restored insulin sensitivity and decreased NAFL and hepatic inflammation compared with HFHCD-fed WT mice. Studies in RCD-fed and HFHCD-fed WT mice revealed augmented protein levels of LIGHT receptors, HVEM and LT β R, and increased hepatic triacylglycerol, inflammatory markers and macrophage content, suggesting a role of LIGHT-dependent signalling in NAFLD induced by HFHCD feeding. Moreover, adipose tissue from HFHCD-fed *Light*^{-/-} mice displayed decreased inflammation and diminished secretion of MCP1, TNF- α and IL-17 cytokines. In agreement with these findings, HFHCD-fed *Light*^{-/-} mice displayed diminished circulating levels of CD115⁺ monocytes, CCR4⁺CCR6⁺ Th9 T cells, IL-6 and TNF- α cytokines, while anti-

inflammatory CD115⁺Ly6C^{low} monocytes were augmented. Hepatic analysis showed reduced content of F4/80⁺ macrophages, CD3⁺ T cells and mRNA levels of the transcription factor *Zbtb16*, determinant for NKT function, a cellular subset associated with liver complications. Similarly, *Light* deficiency in HFHCD-fed *Irs2*^{+/-} mice, which have impaired insulin signalling, also reduced NAFL and inflammation. *Light*^{-/-} mouse liver analysis also revealed a metabolic gene expression signature consistent with improved hepatic homeostasis and reduced mRNA levels of *Klf6* and *Tlr4*, whose expression promotes NAFL-to-NASH and NASH-to-hepatocellular carcinoma (HCC) transitions. These results suggest that *Light* deficiency ameliorates deranged hepatic carbohydrate metabolism, NAFL and inflammation induced by HFHCD. Moreover, *Light* deficiency modulated immune cell phenotype, decreased inflammatory cytokine secretion and

Fig. 7 Analysis of circulating cytokines and leucocyte subsets in WT and *Light*^{-/-} mice fed HFHCD. Circulating plasma levels of (a) MCP1, (b) TNF- α , (c) IL-6, (d) IL-17 and (e) IFN- γ in *Light*^{-/-} and WT mice. (f, h) Percentages of circulating lymphocytes, monocytes and neutrophils identified in the CD45⁺ blood cell population by morphology and by the CD115⁺ monocyte marker and (g, i) CD45⁺CD115⁺Ly6C^{low} and CD45⁺CD115⁺Ly6C^{hi} monocyte subset percentages identified in the CD45⁺CD115⁺ cells in male (f, g) and female (h, i) mice. (j, k) Representative flow cytometry plots of the gating strategy for (j) leucocytes and (k) monocyte subsets in blood samples. Circulating levels of (l) CD4⁺CXCR3⁺ Th1, (m) CD4⁺CCR4⁺CCR6⁻ Th2, (n) CD4⁺CCR4⁺CCR6⁺ Th9 and (o) CD4⁺CCR4⁺CCR6⁺ Th17 cellular subpopulations in WT and *Light*^{-/-} mice. (p) Representative flow cytometry plots of the gating strategy for Th subpopulations in blood samples. Circles, HFHCD-fed mice; white, male WT mice; grey, female WT mice; red, male *Light*^{-/-} mice; blue, female *Light*^{-/-} mice. Data are shown as individual data points and with the mean $\pm$ SEM. Statistical analysis was performed using Student's *t* test (a, d–i, l–o) and Mann–Whitney *U* test (b, c) (**p* < 0.05, ***p* < 0.01 and ****p* < 0.001). SSC, side scatter

diminished pathways associated with NAFL-to-NASH transition, such as KLR6 and toll-like receptor 4 (TLR4). Altogether, these findings point to LIGHT-dependent signaling as a therapeutic target to restore hepatic homeostasis and to alleviate inflammation associated with NAFLD.

Earlier studies have shown elevated circulating LIGHT levels in individuals with insulin resistance/the metabolic syndrome [11] and type 2 diabetes mellitus [12]. We observed improved glucose tolerance in *Light*^{-/-} mice fed HFHCD. Moreover, *Light* deficiency decreased insulin resistance in HFHCD-fed WT mice, suggesting that LIGHT could interfere with insulin sensitivity, as has been shown for other cytokines such as TNF- α [24]. LIGHT exerts important effects on

circulating lipid levels by modulating hepatocyte gene expression and hepatic T lymphocytes [23, 25]. However *Light* deficiency did not modify circulating lipid levels in WT mice.

Notably, in agreement with previous investigations showing enhanced LIGHT levels in human NAFLD [10], *Light* deficiency reduced NAFLD, macrophage and T cell content in HFHCD-fed WT mice. Reduced hepatic inflammation in *Light*^{-/-} mice was accompanied by decreased inflammatory CD11c⁺ macrophages in adipose tissue, which is consistent with reports showing that adipose tissue inflammation signals the progression of NAFLD [22]. Moreover, adipose tissue transplantation from obese mice with a high CD11c⁺ cell content induced hepatic macrophage accumulation and liver

Fig. 8 Hepatic expression in HFHCD-fed WT and *Light*^{-/-} mice. mRNA levels of (a) *Lxr*, (b) *Srebf1c*, (c) *Chrebp*, (d) *Pparg*, (e) *Fasn*, (f) *Ppara*, (g) *Acox*, (h) *Pgc1a*, (i) *Pepck*, (j) *G6pase*, (k) *Cd36*, (l) *Ldlr*, (m) *Abca1*, (n) *Abcg5*, (o) *Abcg8*, (p) *Cyp7a1*, (q) *Klf2*, (r) *Klf4*, (s) *Klf6*, (t) *Klf10* and (u) *Tlr4* in *Light*^{-/-} and WT control mice. mRNA levels were normalised to endogenous cyclophilin expression and are relative to WT mouse levels. Circles, HFHCD-fed mice; white, male WT mice; grey, female WT mice; red, male *Light*^{-/-} mice; blue, female *Light*^{-/-} mice. Data are shown as individual data points with mean $\pm$ SEM. Statistical analysis was performed using Student's *t* test (a–c, e–i, k–u) and Mann–Whitney *U* test (d, j) ($^{\dagger}p = 0.05$, $^*p < 0.05$ and $***p < 0.001$)

damage in high-cholesterol diet-fed *Ldlr*^{-/-} mice [26], which did not develop in RCD-fed mice. These findings suggest that adipose tissue with increased inflammatory CD11c⁺ cells, in the presence of HFHCD, might induce hepatic inflammation and that *Light* deficiency could alleviate inflammation by modulating adipose tissue dysfunction. In support of this hypothesis, LIGHT promotes adipose tissue dysfunction by inhibiting adipogenesis [27], which accommodates excess of fat, and exacerbates adipocyte inflammatory response [28]. In agreement with all of the above, adipose tissue explants from HFHCD-fed *Light*-deficient mice displayed decreased secretion of MCP1, TNF- α and IL-17, whose actions have been related to progression of insulin resistance, liver damage, fibrosis and NASH [29].

The above results indicate that LIGHT (TNFSF14) might alter hepatic homeostasis and function by reducing accumulation of inflammatory cells and inflammatory cytokine secretion. Nevertheless, *Light* deficiency also affected systemic inflammation. HFHCD-fed *Light*^{-/-} mice showed enhanced circulating Ly6C^{low} monocytes and diminished circulating pro-inflammatory Th9 cells and TNF- α and IL-6 cytokine levels. These data might indicate that *Light* deficiency reduces

inflammatory potential of immune cells. However, unexpectedly, augmented secretion of proinflammatory IL-6 and TNF- α cytokines was observed in isolated bone marrow-derived macrophages from *Light*^{-/-} mice compared with WT controls, suggesting an anti-inflammatory role of LIGHT. In addition, immune cells in RCD-fed mice did not display major differences between *Light*^{-/-} and WT mice, with the exception of Th9 which was downregulated in HFHCD conditions but enhanced in RCD-fed *Light*^{-/-} mice. Altogether, this indicates that the immune cell phenotype in our experimental setting with HFHCD feeding is diet dependent and not developmental. On the other hand, in vivo macrophage *Light* deficiency by means of bone marrow transplantation studies showed a protective role of LIGHT (TNFSF14) in a diet-induced obesity model [30]. But, a previous report using a long-term inflammatory choline-deficient high-fat diet demonstrated augmented secretion of LIGHT by hepatic NKT cells during NASH-to-HCC transition, and consequently *Light* deficiency ameliorated disease progression [4]. Consistent with this, *Light* deficiency in HFHCD-fed mice reduced macrophage and T cell hepatic content, hepatic NKT transcription factor expression and NAFLD progression,

suggesting a proinflammatory role of the cytokine by modulating different immune cells. Notably, the finding that LT β R and HVEM expression was markedly augmented, in steatotic and inflamed liver upon HFHCD feeding, indicates that LIGHT-dependent signalling is relevant and induced by stress signals such as the HFHCD feeding. Altogether, this supports the hypothesis that the HFHCD phenotype is partly due to the LIGHT/LT β R-HVEM axis and that defective signalling through this axis restrains NAFLD progression.

Altogether, our findings suggest that LIGHT has a complex role in hepatic metabolic diseases, having divergent effects depending on the experimental setting, diets and the mouse models used. Of note, our study showing that *Light* deficiency confers protection against NAFLD might be highly relevant in clinical scenarios such as a high dietary cholesterol content or in the presence of insulin resistance. Thus, *Light* deficiency in the absence of obesity and in cholesterol-enriched diets might potentially be protective since clinical data in NAFLD in lean individuals provide experimental evidence associating the presence of cholesterol in the diet with progression to NASH [31, 32]. Likewise, *Light* deficiency might also be protective in insulin resistance, since the metabolic syndrome/insulin resistance individuals without obesity, who also display impaired insulin signalling [33], exhibit enhanced circulating LIGHT levels [11]. In this sense, *Light* deficiency decreased hepatic inflammation and steatosis, adipose tissue and systemic inflammation in HFHCD-fed *Irs2*^{+/-} mice, which display impaired insulin signalling and higher hepatic dysfunction than HFHCD-fed WT mice. Thus, therapies targeted to inhibit LIGHT might be of use in these clinical cases.

LT β R-dependent signalling controls lipid homeostasis by modulating gene expression in the liver [23]. Analysis of lipogenic, β -oxidation and gluconeogenic genes did not consistently show improvement of these pathways in *Light*-deficient mice. Thus, protective *Pparg* and *Ppara* gene mRNA expression levels were unexpectedly decreased while mRNA levels of *Srebf1c*, *Ldlr* and *Fasn* related to lipogenesis and lipid uptake were higher, which could alternatively indicate a compensatory mechanism induced by reduced hepatic lipid pool. In fact, *Light*-deficient mice had diminished *Lxr* and *Abca1*, whose activities are associated with aberrant lipid storage and accumulation of hepatic cholesterol pool in NAFLD [34, 35]. Nevertheless, additional studies are required to fully understand the metabolic regulation of hepatic LIGHT-mediated signalling. Consistent with improved NAFL, *Light* deficiency decreased important hepatic genes associated with liver damage such as *Zbtb16* [4, 36], a transcription factor determinant for NKT cells, as well as *Klf6* and *Tlr4*, whose function is highly relevant in the NAFL-to-NASH transition, hepatic stellate cell activation and HCC [36, 37]. Altogether, these findings indicate that *Light* deficiency might reduce NAFL progression by modulating relevant hepatic genes involved in NAFLD.

In summary, the present study showed that *Light* deficiency in HFHCD mice improves glucose tolerance and insulin sensitivity, and reduces NAFL, hepatic inflammation and adipose tissue proinflammatory cytokine secretion. In addition, *Light* deficiency produced a hepatic expression signature consistent with improved hepatic homeostasis and conferred protection against inflammation associated with NAFL-to-NASH transition by diminishing the hepatic expression of relevant actors in this pathologic process, *Klf6* and *Tlr4* genes. These results suggest that blocking LIGHT-dependent signalling might be of use in NAFLD in insulin resistance states and in NAFLD caused by cholesterol-enriched diets.

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Data availability All data generated or analysed during this study are included in the published article (and its additional files). Data regarding the manuscript are available upon reasonable request made to the corresponding author.

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Contribution statement AH-C and AV acquired and analysed all of the data, participated in the interpretation of results and helped to write the manuscript. DJB participated in study design, writing and critical revision of the manuscript. HG-N conceived and designed the study, supervised the data acquisition, interpreted the results and wrote the manuscript. All authors gave final approval of the version to be published. HG-N is the guarantor of this work.

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