Research Article

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Toxic lipids stored by Kupffer cells correlates with their pro-inflammatory phenotype at an early stage of steatohepatitis

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In memory of Professor Dominique Emilie.

Background & Aims: Non-alcoholic steatohepatitis (NASH) is characterized by steatosis associated with liver inflammation. Steatosis causes recruitment of lymphocytes into the liver and this is worsened by lipopolysaccharides (LPS). As macrophages may be involved in the lymphocyte homing, we studied the role of lipids in determining the phenotype of Kupffer cells (KCs) at the stage of steatosis.

Methods: Steatosis was induced in mice by a high fat diet. The turnover and the recruitment of KCs were analyzed *in vivo* by flow cytometry. KCs phenotype was assessed by optical and electron microscopy, cell culture and lymphocyte recruitment by *in vitro* chemotaxis. Lipidomic analysis was carried out by mass-spectrometry and gene expression analysis by TaqMan low density array.

Results: Although the number of KCs was not modified in steatotic livers compared to normal livers, their phenotypes were different. Electron microscopy demonstrated that the KCs from fatty livers were enlarged and loaded with lipid droplets. Lipid synthesis and trafficking were dysregulated in fat-laden KCs and toxic lipids accumulated. Fat-laden KCs recruited more CD4+ T and B lymphocytes in response to LPS stimulation than did control KCs and produced high levels of pro-inflammatory cytokines/chemokines, which could be reversed by inhibition of lipogenesis.

Conclusions: Lipid accumulation in fat-laden KCs is due to a dysregulation of lipid metabolism and trafficking. Fat-laden KCs are "primed" to recruit lymphocytes and exhibit a pro-inflammatory phenotype, which is reversible with inhibition of lipogenesis.

Keywords: NASH; Kupffer cells; Inflammation; Lipidomic; Obesity.

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Abbreviations: NASH, non-alcoholic steatohepatitis; LPS, lipopolysaccharide; KC, Kupffer cell; NAFLD, non-alcoholic fatty liver disease; TNFα, tumor necrosis factor-α; IFNγ, interferon-γ; IL, interkeukin; ND, normal diet; HFD, high fat diet; TG, triacylglycerol; NPC, non-parenchymateous cells; TOFA, 5-(tetradecyloxy)-2-furoic acid; TLDA, TaqMan low density array; OGTT, oral glucose tolerance test; HOMA, homeostasis model assessment of insulin-resistance; ALT, alanine aminotransferase; CCL, C-C motif chemokine ligand; ATM, adipose tissue macrophages; FA, fatty acid; FAS, fatty acid synthase; ACC1, acetyl-CoA carboxylase 1; ChREBP, carbohydrate-responsive element binding protein; PPAR, peroxisome proliferator-activated receptor; FABP, fatty acid-binding protein; FATP5, fatty acid transport protein 5; DGAT, diacylglycerol acyltransferase; SCD1, stearoyl-CoA desaturase 1; CPT1A, carnitine parmitoyltransferase 1A; HMG-CoA, β-hydroxy-β-methylglutaryl coenzyme A; ACAT1, acyl-CoA cholesterol acyltransferase 1; CE, cholesterol esters; ABCA6, ATP binding cassette transporter A6; MARCO, macrophage receptor with collagenous structure; LDL, low-density lipoprotein; TLR, toll like receptors; MTP, microsomal triglyceride transfer protein; VLDL, very low-density lipoprotein; LXRα, liver X receptors alpha; MSR1 (SR-AI), macrophage scavenger receptor; FC, free cholesterol; DAG, diacylglycerol; CXCL10, C-X-C motif chemokine 10; SREBP1c, sterol regulatory element binding proteins isoform 1c; SR-PSOX, scavenger receptor binding phosphatidylserine and oxidized lipoprotein.

Introduction

The prevalence of non-alcoholic fatty liver disease (NAFLD) is increasing worldwide and is linked to the expansion of obesity and type 2 diabetes [1]. NAFLD ranges from pure steatosis to non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis, and hepatocellular carcinoma [2]. Obesity with or without associated insulin resistance is largely involved in excessive fat accumulation in the liver [3]. Although weight loss and correction of insulin resistance are relevant therapeutic targets to improve NASH [4], there is currently no treatment for advanced NAFLD [3]. In this regard, understanding the mechanisms responsible for the inflammatory processes leading to NASH remains a significant challenge.

Recruitment of inflammatory cells into the liver and their subsequent activation are key steps in the progression of liver disease. NAFLD is associated with alterated hepatic lymphocyte subsets [5] including reduced numbers of hepatic NKT lymphocytes and T regulatory lymphocytes. Correcting these NKT or T regulatory lymphocyte deficiencies by adoptive transfer in

murine models of NASH reduces liver damage [6,7]. In addition to the redistribution of hepatic lymphocyte subsets, we have shown that liver inflammation in obese mice results not only from steatosis but also from lymphocyte hyper-responsiveness to chemotactic agents [8].

The liver contains an array of potential antigen-presenting cells, including Kupffer cells (KCs), which could participate in the redistribution of hepatic lymphocyte subsets during NAFLD. KCs constitute 20–25% of the non-parenchymal cells in the liver. KCs are the primary source of hepatic pro-inflammatory cytokines, such as tumor necrosis factor-α (TNFα) [9]. In addition to their essential role as phagocytes, KCs participate in liver T cell tolerance [10]. Consequently, modified KC phenotypes could be involved in an altered immune response by disrupting T cell tolerance in the liver. Macrophage phenotypes are based on the presence of specific receptors and on the cytokines and chemokines they produce, classifying them into two subsets defined as M1 and M2 [11]. M1 macrophages are induced by pro-inflammatory mediators, such as lipopolysaccharide (LPS) or interferon-γ (IFNγ), and produce pro-inflammatory cytokines and chemokines. Conversely, M2 macrophages are stimulated by interleukin (IL) 4 and IL13 and this stimulation induces low levels of pro-inflammatory cytokines and high levels of IL10. In a recent study that addressed the role of lipid metabolism in macrophage activation, lipogenesis was found to be a pre-requisite for macrophage activation [12]. We therefore aimed at studying the involvement of lipids in determining KC phenotypes at the step of steatosis.

Materials and methods

Animals and diets

C57BL/6J and *ob/ob* mice were purchased from Janvier (France) and maintained under a 12-h light/dark schedule, with food and water *ad libitum* and treated in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). Mice were fed either a normal diet (ND) or a high-fat diet (HFD) (Safe, France) as described in Supplementary Materials.

Lipidomic analysis

Hepatic triacylglycerols (TGs) were extracted with a chloroform–methanol mixture and separated by thin layer chromatography. TGs were then extracted from the silica plate with acetone and measured with a colorimetric diagnostic kit (Triglycerides FS; Diasys). For lipidomic analysis, the liver and KCs were homogenized in 1 ml of methanol/water (2:1, v/v) containing 5 mM EGTA. Lipids were extracted and molecular species were quantified by gas liquid chromatography as previously described [13].

Isolation and culture of KCs

Livers were homogenized and non-parenchymal cells (NPC) and KCs were recovered as described in Supplementary Materials. NPC were resuspended in PBS 2% FCS prior to staining with mAbs and flow cytometry analysis. KCs (10^6 cells) were plated in RPMI, 10% FCS (PAA, Austria) and 1% penicillin/streptomycin for 24 h and then stimulated with 100 ng/ml LPS from *Escherichia coli* serotype O55:B5 endotoxin-free (Alexis, Switzerland) or not for 24 h. For TOFA (5-(tetradecyloxy)-2-furoic acid) (Merrel National Laboratories, USA) exposure, KCs were treated with 200 μM TOFA for 24 h. KC culture supernatants were stored frozen or used in an *in vitro* chemotaxis assay. Cell viability was evaluated by trypan blue and was higher than 90%. There was no contamination by hepatocytes. The purity was assessed by F4/80, CD31, and CD11c labeling and ranged from 85% to 89% with some contamination by endothelial cells (4.8%) or dendritic cells (2.4%) (data not shown).

Liver monocyte recruitment

Aggregates of PKH26-PCL (0.1 mM; Sigma, MO) or saline were intravenously injected into mice fed with HFD or ND at the age of 4 weeks. Mice fed the HFD and ND diets were sacrificed 4, 8, 12, and 16 weeks after the PKH26-PCL or saline injection. Macrophages were stained with a rat anti-F4/80 mAb (AbD Serotec, UK) and analyzed using a FACSCalibur cytometer (Becton Dickinson, USA).

Lymphocyte recruitment assay

Lymphocyte chemotaxis was evaluated using a Transwell® system (5 μm pores, Corning Costar, MA). Splenocytes from lean mice were purified by centrifugation through a Lympholyte® density gradient (Cedarlane, Canada) at 800 g for 20 min. Lymphocytes were stained with anti-CD3, anti-CD4, anti-CD8, and anti-CD19 mAbs. KC supernatants were placed in the lower chamber and 1.5×10^6 lymphocytes in 150 μl were placed into the upper chamber (same culture medium in both chambers). Uncultured medium in the lower chamber with 1.5×10^6 cells in the upper chamber were used as input control. After 4 h of incubation, cells in the lower chamber were analyzed by flow cytometry. To quantify the lymphocytes subsets infiltrating the liver, anti-CD45 or antibodies described above were used. NPC were stained and we counted the number of labeled lymphocytes per g of liver.

RNA and TaqMan low-density array analysis (TLDA)

RNAs were extracted using the RNeasy Lipid Tissue kit (Qiagen, CA) including a DNase treatment. TLDA was performed following the manufacturer's instructions and described in Supplementary Materials.

Statistical analysis

All analyses were performed using the StatView (version 5.0 statistical software system (Abacus Concepts, CA).

Results

HFD-induced steatosis does not modify recruitment of KCs

To determine whether KCs were involved in the early steps of NASH, C57BL/6 J mice were fed the HFD for 16 weeks. This diet induced a mean weight gain of 26% (±4%) and obesity-related metabolic disorders (Table 1). Insulinemia, blood glucose levels, oral glucose tolerance test (OGTT) results and calculated homeostasis model assessment of insulin-resistance (HOMA) clearly demonstrated that the HFD mice were insulin-resistant. The plasma levels of TGs, leptin, resistin, and CCL2 were significantly higher in HFD mice compared to those of ND mice. The HFD mouse liver exhibited clear signs of steatosis and had TG levels that were fourfold higher than the liver TG levels of ND mice (Fig. 1A and B). Alanine aminotransferase (ALT) levels were not significantly modified by the HFD diet (Table 1). There was no liver inflammation in HFD mice, as shown by the absence of inflammatory infiltrates at histological examination and by the numbers of liver CD45 lymphocytes on flow cytometry analysis, which were not higher than those of ND mice (Fig. 1 A and C). Therefore, HFD mice were at an early step of NAFLD, with a fatty liver but no significant inflammation.

To study the number of KCs and their recruitment to the liver during steatosis development, we injected mice intravenously with aggregates of PKH26 [14]. After 24 h, more than 95% of KCs were PKH26+ and all monocytes were PKH26− (data not shown). Mice were then maintained on HFD or ND for 4, 8, 12, or 16 weeks. Recruitment of new liver macrophages was assessed by the number of $PKH26^-/F4/80^+$ cells found in the liver. The