Research Article

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# Hematopoietic overexpression of Cyp27a1 reduces hepatic inflammation independently of 27-hydroxycholesterol levels in *Ldlr*$^{-/-}$ mice

Tim Hendrikx[1,2], Mike L.J. Jeurissen[1,2], Veerle Bieghs[1,2], Sofie M.A. Walenbergh[1,2], Patrick J. van Gorp[1,2], Fons Verheyen[1,2], Tom Houben[1,2], Yasmin Dias Guichot[1,2], Marion J.J. Gijbels[1,2], Eran Leitersdorf[3], Marten H. Hofker[4], Dieter Lütjohann[5], Ronit Shiri-Sverdlov[1,2,*]

[1]*Department of Molecular Genetics, ELMI Unit (CRISP) and Pathology, Nutrition and Toxicology Research (NUTRIM) and Cardiovascular Research (CARIM), Institutes of Maastricht, University of Maastricht, Maastricht, The Netherlands;* [2]*Department of Molecular Cell Biology, ELMI Unit (CRISP) and Pathology, Nutrition and Toxicology Research (NUTRIM) and Cardiovascular Research (CARIM), Institutes of Maastricht, University of Maastricht, Maastricht, The Netherlands;* [3]*Department of Medicine, Hadassah-Hebrew University Medical Center, Jerusalem, Israel;* [4]*Department of Pathology & Laboratory Medicine, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands;* [5]*Institute of Clinical Chemistry and Clinical Pharmacology, University of Bonn, Bonn, Germany*

**Background & Aims**: Non-alcoholic steatohepatitis (NASH) is characterized by hepatic lipid accumulation and inflammation. Currently, the underlying mechanisms, leading to hepatic inflammation, are still unknown. The breakdown of free cholesterol inside Kupffer cells (KCs) by the mitochondrial enzyme CYP27A1 produces 27-hydroxycholesterol (27HC). We recently demonstrated that administration of 27HC to hyperlipidemic mice reduced hepatic inflammation. In line, hematopoietic deletion of *Cyp27a1* resulted in increased hepatic inflammation. In the current manuscript, the effect of hematopoietic overexpression of Cyp27a1 on the development of NASH and cholesterol trafficking was investigated. We hypothesized that Cyp27a1 overexpression in KCs will lead to reduced hepatic inflammation.

**Methods**: Irradiated *Ldlr*$^{-/-}$ mice were transplanted (tp) with bone marrow from mice overexpressing Cyp27a1 (*Cyp27a1*$^{over}$) and wild type (Wt) mice and fed either chow or a high-fat, high-cholesterol (HFC) diet for 3 months. Additionally, gene expression was assessed in bone marrow-derived macrophages (BMDM) from *Cyp27a1*$^{over}$ and Wt mice.

**Results**: In line with our hypothesis, hepatic inflammation in HFC-fed *Cyp27a1*$^{over}$-tp mice was reduced and KCs were less foamy compared to Wt-tp mice. Remarkably, these changes occurred even though plasma and liver levels of 27HC did not differ between both groups. BMDM from *Cyp27a1*$^{over}$ mice revealed reduced inflammatory gene expression and increased expression of cholesterol transporters compared to Wt BMDM after lipopolysaccharide (LPS) stimulation.

**Conclusions**: Our data suggest that overexpression of Cyp27a1 in KCs reduces hepatic inflammation independently of 27HC levels in plasma and liver, further pointing towards KCs as specific target for improving the therapy of NASH.



## Introduction

Non-alcoholic fatty liver disease (NAFLD) is considered the hepatic event of the metabolic syndrome and is characterized by the deposition of fat in the liver (steatosis). NAFLD covers a broad spectrum of diseases ranging from steatosis to non-alcoholic steatohepatitis (NASH). NASH is distinguished from simple steatosis by the added presence of inflammation in the liver. Whereas steatosis is generally considered a relatively benign and reversible condition, inflammation adversely affects the long-term prognosis of liver diseases as this enables the development of more advanced stages of the disease, including fibrosis, cirrhosis or hepatocellular carcinoma, ultimately requiring liver transplantation [1]. So far, the intracellular mechanisms that trigger the inflammatory response are not known. Hence, therapy options are very poor and lack specificity.

The uptake of dietary cholesterol by Kupffer cells (KCs), the resident macrophages of the liver, was found to play an important role during NASH development [2]. Similar to previously reported observations during atherosclerosis [3,4], the

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* Corresponding author. Address: Department of Molecular Genetics, Maastricht University, P.O. Box 616, 6200MD Maastricht, The Netherlands. Tel.: +31 43 388 1746; fax: +31 43 388 4574.
*E-mail address:* r.sverdlov@maastrichtuniversity.nl (R. Shiri-Sverdlov).

*Abbreviations:* NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; KC, Kupffer cell; 27HC, 27-hydroxycholesterol; Ldl, low density lipoprotein receptor; HFC, high-fat high-cholesterol; Wt, wild type; tp, transplanted; TG, triglycerides; FFA, free fatty acids; BMDM, bone marrow derived macrophages; LPS, lipopolysaccharide; NPC, Niemann-Pick type C.

accumulation of cholesterol leading to a swollen appearance of macrophages, termed foam cells, was associated with an increased inflammatory response in the liver [5]. Upon uptake by macrophages, cholesterol is initially directed to lysosomes for hydrolyzation and then further transported to the cytoplasm. Here, cholesterol can be converted into 27-hydroxycholesterol (27HC) by the action of the mitochondrial enzyme CYP27A1 as the first step in the alternative pathway of bile acid formation [6]. Recently, we demonstrated that exogenous administration of 27HC dramatically reduced hepatic inflammation in hyperlipidemic $Ldlr^{-/-}$ mice upon high-fat, high-cholesterol (HFC) feeding [7]. In line with this observation, hematopoietic deletion of *Cyp27a1* resulted in increased hepatic inflammation [7].

We hypothesized that hematopoietic overexpression of Cyp27a1 will lead to reduced hepatic inflammation. In order to investigate the effect of overexpression of Cyp27a1 in KCs on hepatic inflammation, bone marrow chimeras were generated by injecting bone marrow cells from mice overexpressing Cyp27a1 ($Cyp27a1^{over}$) into lethally irradiated $Ldlr^{-/-}$ hyperlipidemic host mice. In the current study we show that overexpression of Cyp27a1 in KCs reduces hepatic inflammation, independently of hepatic and plasma 27HC levels.

## Materials and methods

### *Bone marrow-derived macrophages*

Bone marrow-derived macrophages were isolated from the tibiae and femurs of C57BL/6 or $Cyp27a1^{over}$ mice (kindly provided by E. Leitersdorf [8]). Cells were cultured in RPMI-1640 (GIBCO Invitrogen, Breda, the Netherlands) with 10% heat-inactivated foetal calf serum (Bodinco B.V. Alkmaar, the Netherlands), penicillin (100 U/ml), streptomycin (100 μg/ml) and L-glutamine 2 mM (all GIBCO Invitrogen, Breda, the Netherlands), supplemented with 20% L929-conditioned medium (LCM) for 8–9 days to generate bone marrow-derived macrophages. After attachment, macrophages were seeded at 350,000 cells per well in 24-well plates and incubated for 24 h with medium (control), cyclodextrin (carrier control) or 27HC (0.25 μM; 1 μM). Then cells were washed and stimulated with LPS (100 ng/ml) for 4 h. Finally, cells were lysed for mRNA expression analysis. For protein expression analysis and electron microscopy analysis, cells were seeded at 2,000,000 cells per well in 6-well plates and incubated under the same conditions.

### *Mice, diet, and bone marrow transplantation*

Mice were housed under standard conditions and given free access to food and water. Experiments were performed according to the Dutch regulations and approved by the Committee for Animal Welfare of the Maastricht University. Female 12-week-old $Ldlr^{-/-}$ mice were lethally irradiated and transplanted with Wt or $Cyp27a1^{over}$ bone marrow as previously described [9]. After a recovery period of 9 weeks, the mice were given either chow or HFC diet for 3 months (chow: n = 5; HFC: n = 10). The HFC diet contained 21% milk butter, 0.2% cholesterol, 46% carbohydrates, and 17% casein. Collection of blood and tissue specimens, biochemical determination of lipids in plasma and liver, liver histology, electron microscopy, RNA isolation, cDNA synthesis, qPCR and oxysterol levels were determined as described previously [7,10].

### *Statistical analysis*

Data were analysed using the Graphpad Prism 4.0.3 software. Groups were compared using the unpaired *t* test for comparing two groups or one-way ANOVA for comparing multiple groups. Data were expressed as the mean and standard error of the mean and were considered significantly different at $^{*}p < 0.05$; $^{**}p < 0.01$; or $^{***}p < 0.001$.

## Results

### *$Cyp27a1^{over}$-tp mice have less hepatic inflammation compared to Wt-tp mice*

The effect of hematopoietic overexpression of Cyp27a1 in diet-induced NASH was investigated by transplanting bone marrow from wild type (Wt) and Cyp27a1 overexpressing ($Cyp27a1^{over}$) mice into $Ldlr^{-/-}$ mice. After a recovery period of 9 weeks, mice received chow or HFC diet for 3 months. Body weight did not differ significantly between the groups (data not shown). To investigate the effect of hematopoietic overexpression of Cyp27a1 on hepatic inflammation, liver sections were stained with antibodies against several inflammatory markers including macrophages and neutrophils. Lower numbers of infiltrating macrophages ($p = 0.0206$) and neutrophils ($p = 0.0146$) were observed in the livers of $Cyp27a1^{over}$-tp mice compared to Wt-tp mice after HFC (Fig. 1A), as further illustrated by representative pictures from Mac-1 staining for infiltrating macrophages and neutrophils (Fig. 1B). These findings were confirmed by reduced hepatic gene expression of the monocyte chemo-attractant protein 1 (*Mcp1*) ($p = 0.0083$), chemokine (C-X-C motif) ligand 1 (*Cxcl1*) ($p = 0.046$), and *Cxcl2* ($p = 0.039$) in $Cyp27a1^{over}$-tp mice compared to Wt-tp mice upon HFC (Fig. 1C), whereas gene expression for tumor necrosis factor-alpha (*Tnfα*) showed the same trend, although it did not reach significance ($p = 0.07$). Taken together, these data indicate that hematopoietic overexpression of Cyp27a1 reduces hepatic inflammation.

To investigate the effect of overexpression of Cyp27a1 in hematopoietic cells on apoptosis, hepatic expression of genes important during apoptosis was determined. Compared to animals on chow, expression of the apoptotic genes *Bfl1* and *Traf1* was increased after 3 months of HFC diet. However, no difference was observed between Wt-tp and $Cyp27a1^{over}$-tp mice (Supplementary Fig. 1A). In line with these findings, no difference between Wt-tp and $Cyp27a1^{over}$-tp mice was found in hepatic expression of catalase (*Cat*), *SOD2*, *Hmox*, and *Cyp2E1*, markers for oxidative stress (Supplementary Fig. 1B). To further characterize these two genotypes, markers for liver damage and fibrosis were analysed. As expected, plasma alanine transaminase (ALT) levels were increased in mice after 3 months of HFC feeding. Similar ALT levels were observed in Wt-tp and $Cyp27a1^{over}$-tp mice. Additionally, hepatic gene expression of *Tgfβ*, a marker for fibrosis development, was unchanged between Wt-tp and $Cyp27a1^{over}$-tp mice upon HFC diet (Supplementary Fig. 1C). To evaluate macrophage polarization in the livers of both transplanted groups, hepatic gene expression analysis of *IL12*, an M1 macrophage marker, was measured and revealed no difference between the two groups. Likewise, no difference was observed in the expression of the M2 macrophage markers arginase-1 (*Arg1*) and *IL10* after 3 months of HFC feeding (Supplementary Fig. 1D). Taken together, these data indicate that hematopoietic overexpression of Cyp27a1 reduces hepatic inflammation independent of the level of apoptosis, oxidative stress, liver damage or macrophage subset polarization.

### *Levels of 27-hydroxycholesterol in liver and plasma are not affected by hematopoietic Cyp27a1 overexpression*

After three months of HFC diet, no difference was found between the transplanted groups with regard to hepatic levels of