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Research Article

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

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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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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

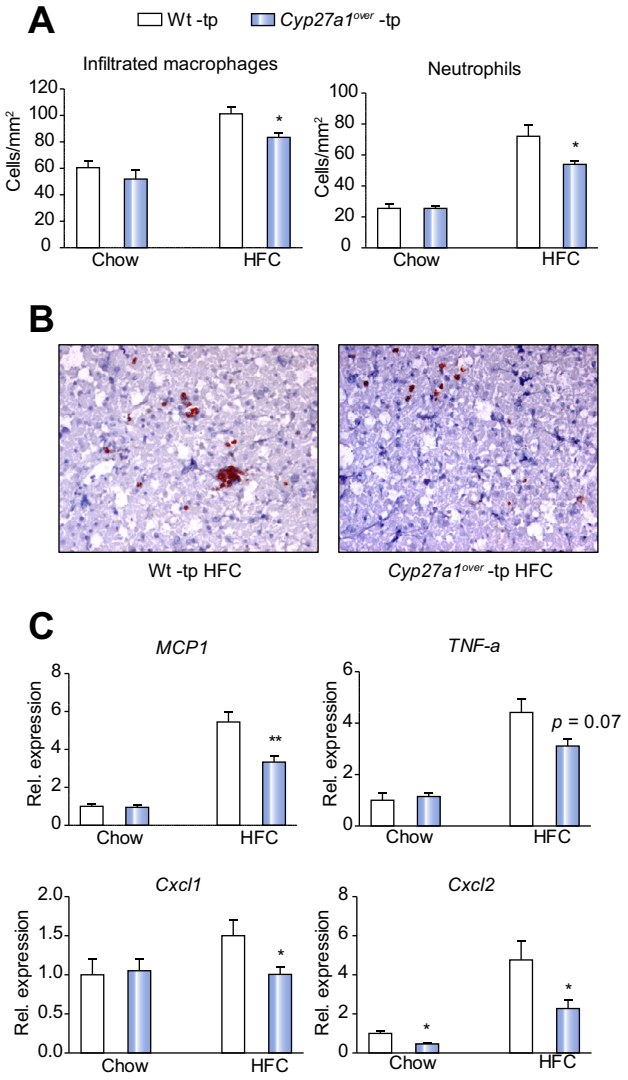

Fig. 1. Parameters of hepatic inflammation in Wt-tp and *Cyp27a1*^{over}-tp mice. (A) Liver sections were stained for infiltrating macrophages and neutrophils (Mac-1), neutrophils (NIMP) and positive cells were counted. (B) Representative pictures of Mac-1 staining (200× magnification) after 3 months of HFC diet in Wt-tp and *Cyp27a1*^{over}-tp mice. (C) Gene expression analysis of monocyte chemoattractant protein 1 (*Mcp1*), tumor necrosis factor alpha (*Tnfa*), chemokine (C-X-C motif) ligand 1 (*Cxcl1*) and *Cxcl2*. Gene expression data were set relative to Wt-tp mice on chow diet. *Indicates $p < 0.05$ and **$p < 0.01$. (This figure appears in colour on the web.)

triglycerides (TGs), cholesterol and free fatty acids (FFAs) (Supplementary Fig. 2A). In addition, while plasma lipid levels were increased in mice receiving HFC for 3 months, hematopoietic overexpression of Cyp27a1 did not affect plasma lipid levels. Total cholesterol, TG and FFA levels in plasma were not different in *Cyp27a1*^{over}-tp mice compared to their controls, both in mice on chow as well as on HFC (Supplementary Fig. 2B). Altogether, hematopoietic overexpression of Cyp27a1 does not affect plasma and liver lipid levels.

In order to study the effect of Cyp27a1 overexpression on 27-hydroxycholesterol levels in hematopoietic cells, 27-hydroxycholesterol in liver and plasma was measured in Wt-tp and *Cyp27a1*^{over}-tp mice. Surprisingly, no difference in the levels of 27-hydroxycholesterol in liver and plasma between both transplanted groups were observed (Fig. 2A).

Additionally, in order to investigate whether hematopoietic overexpression of Cyp27a1 is related to changes in the level of other oxysterols, levels of 7α- and 24-hydroxycholesterol in liver and plasma were measured. There were no differences observed in 7α- and 24-hydroxycholesterol levels in the livers and plasma of *Cyp27a1*^{over}-tp mice compared to Wt-tp mice (Fig. 2B). Taken together, these data indicate that the observed anti-inflammatory effect is not related to changes in oxysterol levels in liver and plasma.

To further investigate the similarity in systemic and hepatic levels of 27HC between the two groups, gene expression analysis of hepatic *Cyp7b1*, *Cyp7a1*, and *Cyp27a1* was performed. No difference in the expression of *Cyp7b1* between Wt-tp and Cyp27a1^{over}-tp mice was found, suggesting that degradation of 27HC is not different between the groups (Fig. 2C). Hepatic gene expression levels of *Cyp7a1* were increased in *Cyp27a1*^{over}-tp mice compared to control mice (Fig. 2C), suggesting that more cholesterol is broken down in the classic pathway and therefore production of hepatic and systemic 27HC is not significantly increased in *Cyp27a1*^{over}-tp mice. Furthermore, gene expression of *Cyp27a1* in total liver was not different between Wt-tp and *Cyp27a1*^{over}-tp mice (Fig. 2C). Altogether, these data suggest that the increased systemic and hepatic production of 27HC is prevented by upregulation of the classical pathway.

In order to further evaluate possible protective mechanisms of Cyp27a1 overexpression, the amount of free cholesterol and cholesteryl esters in the liver were measured. Results of these measurements show that the amount of free cholesterol in livers of *Cyp27a1*^{over}-tp mice is not different from the amount in Wt-tp mice (Supplementary Fig. 3A). Interestingly, hepatic levels of cholesteryl esters were dramatically reduced in *Cyp27a1*^{over}-tp mice compared to the levels in Wt-tp mice (Supplementary Fig. 3A). In order to investigate any difference in the production of cholesteryl esters, gene expression analysis of *ACAT2* was performed. Although there was a trend towards a reduction, compared to Wt-tp mice upon HFC, hepatic *ACAT2* expression was unchanged in mice overexpressing Cyp27a1 in hematopoietic cells (Supplementary Fig. 3B). Taken together, these data indicate that Cyp27a1 overexpression can protect from hepatic inflammation by reduced accumulation of cholesteryl esters in the liver, without affecting cholesteryl production by ACAT2.

Less foamy Kupffer cells in Cyp27a1^{over}*-tp mice compared to Wt-tp mice*

To analyse the effect of Cyp27a1 overexpression on the foamy appearance of KCs, a CD68 staining (for KC) was performed and revealed a clear difference between *Cyp27a1*^{over}-tp mice and Wt-tp mice upon HFC diet. In *Cyp27a1*^{over}-tp mice, KCs are less swollen and foamy compared to KCs in Wt-tp animals (Supplementary Fig. 4A). These data are in line with reduced gene expression of *Cd68* in the livers of *Cyp27a1*^{over}-tp mice compared to Wt-tp mice after 3 months of HFC feeding (Supplementary Fig. 4B).

To investigate whether the difference in foamy appearance of KCs is related to changes in cholesterol uptake or cholesterol efflux, gene expression analysis was performed. For cholesterol uptake, hepatic expression of *Cd36*, scavenger receptor a (*SR-A*), low density lipoprotein receptor-related protein 1 (*LRP1*) and

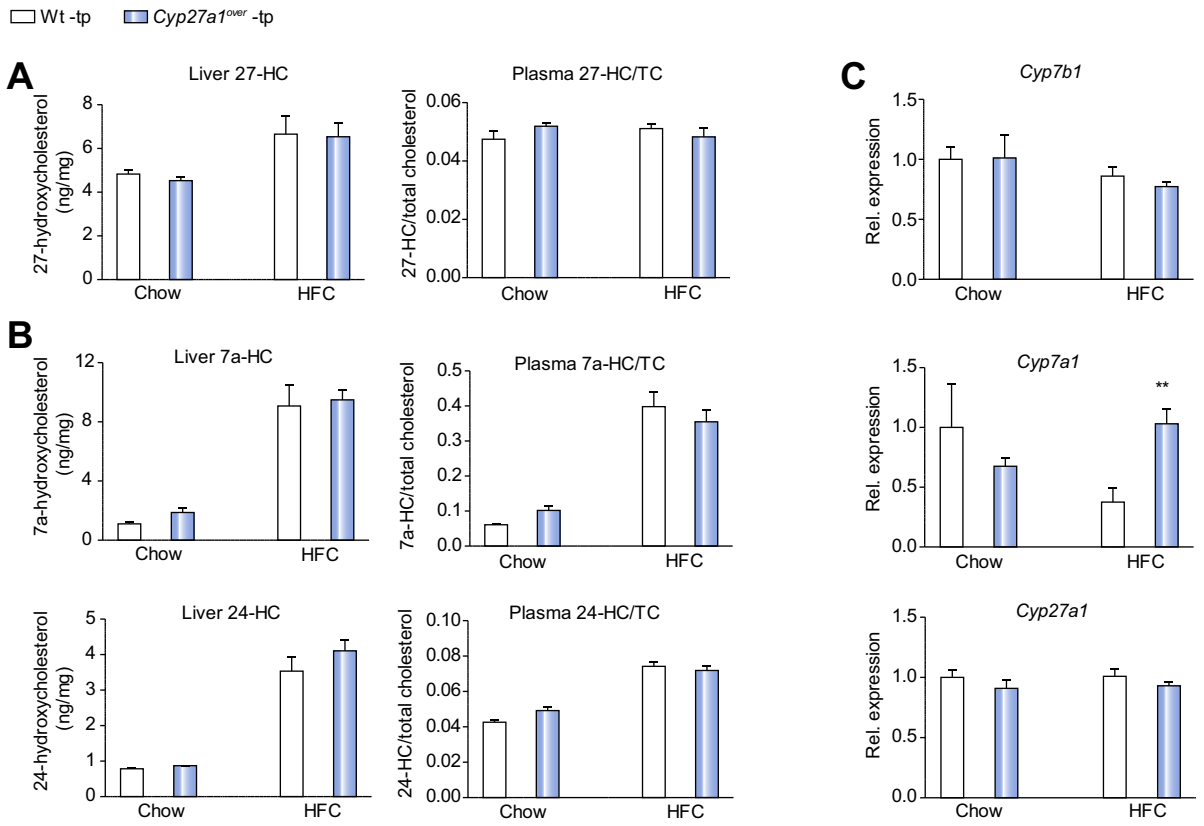

Fig. 2. Liver and plasma oxysterol levels in Wt-tp and *Cyp27a1*^{over}-tp mice. (A) Liver and plasma 27-hydroxycholesterol levels after chow and HFC diet. (B) Liver and plasma 7a-hydroxycholesterol and 24-hydroxycholesterol levels after chow and HFC diet. (C) Gene expression analysis of hepatic *Cyp7b1*, *Cyp7a1*, and *Cyp27a1* in Wt-tp and *Cyp27a1*^{over}-tp mice. Gene expression data were set relative to Wt-tp mice on chow diet. *Indicates p <0.05.

SR-B1 did not differ between Wt-tp and *Cyp27*^{over}-tp mice upon HFC (Supplementary Fig. 4C). Next to that, no difference in hepatic expression of two well-known cholesterol efflux transporters, *Abca1* and *Abcg1*, was observed between Wt-tp and *Cyp27*^{over}-tp mice (Supplementary Fig. 4D). Together, these data indicate that changes in the foamy appearance of KCs are not related to cholesterol uptake and reverse cholesterol transport in total liver.

Bone marrow-derived macrophages (BMDM) from Cyp27a1^{over} *mice have increased intracellular cholesterol trafficking*

In order to investigate the mechanism by which Cyp27a1 reduces inflammation, bone marrow cells were isolated from Wt and *Cyp27a1*^{over} mice and cultured to macrophages. After stimulation with lipopolysaccharide (LPS) for 4 h, the expression of *Tnfα* was significantly lower in BMDM from *Cyp27a1*^{over} mice compared to those from Wt mice, confirming our *in vivo* findings that Cyp27a1 overexpression in macrophages results in a reduced inflammatory response (Fig. 3A).

To study the specific effect of Cyp27a1 overexpression on cholesterol uptake by macrophages, the expression of *Cd36* and *SR-A* was analysed with and without LPS stimulation. No difference was observed between BMDM from Wt and from *Cyp27a1*^{over} mice (Fig. 3B). On the other hand, expression of liver X receptor alpha (*LXRα*) ($p = 0.025$), *Abca1* ($p = 0.0094$), and *Abcg1* ($p = 0.0462$), genes involved in cholesterol efflux, was increased in BMDM from *Cyp27a1*^{over} mice compared to Wt BMDM after LPS stimulation (Fig. 3C). These data indicate that Cyp27a1

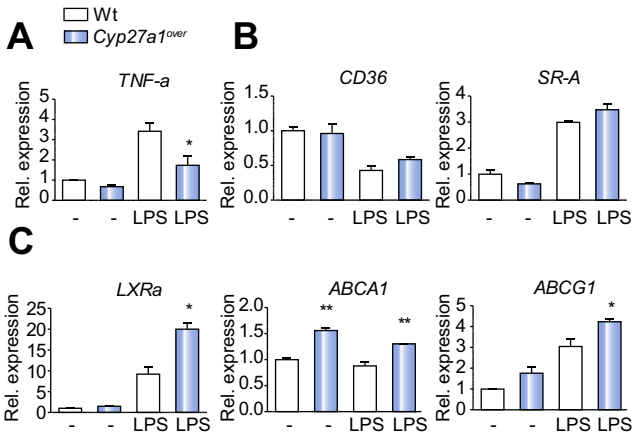

Fig. 3. Gene expression in bone marrow-derived macrophages from Wt and *Cyp27a1*^{over} mice after LPS stimulation. Gene expression analysis of the inflammatory marker tumor necrosis factor alpha (*Tnfα*) (A), scavenger receptors *Cd36* and *SR-A* (B), liver X receptor alpha (*LXRα*) and ATP-binding cassette transporter A1 (*Abca1*) and G1 (*Abcg1*) (C) in BMDM from Wt and *Cyp27a1*^{over} mice after 4 h LPS stimulation. Data were set relative to BMDM from Wt mice incubated with medium. *Indicates p <0.05 and **p <0.01.

overexpression in macrophages leads to an increase of cholesterol efflux transport during an inflammatory response.

To further examine the effect of Cyp27a1 overexpression on intracellular cholesterol trafficking in macrophages, gene

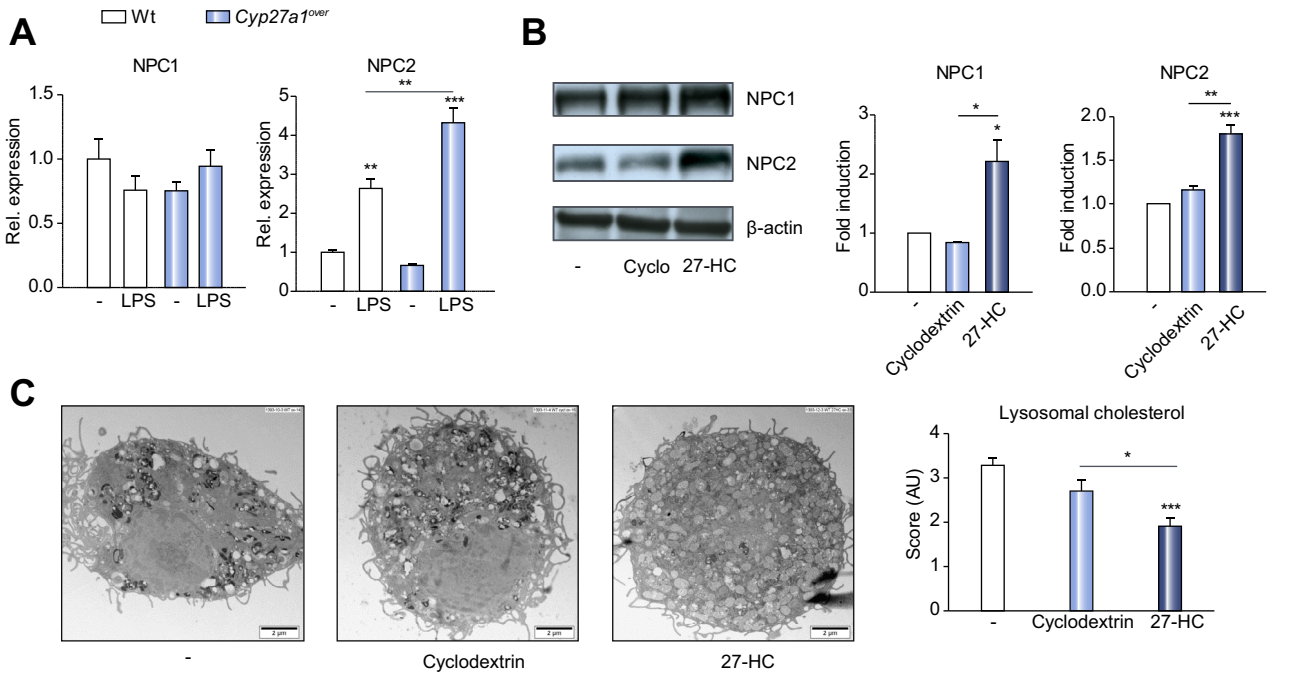

Fig. 4. Gene expression and protein levels in bone marrow-derived macrophages from Wt and *Cyp27a1*^{over} mice of genes important in intracellular cholesterol trafficking. (A) Niemann-Pick C1 (*Npc1*) and *Npc2* gene expression in BMDM from Wt and *Cyp27a1*^{over} mice after 4 h LPS stimulation. (B) Representative pictures of 5 independent Western blot measurements. Western blot analysis of *Npc1* and *Npc2* proteins in BMDM from Wt mice after 4 h of LPS stimulation and incubation with cyclodextrin (carrier control) and 27-hydroxycholesterol. Beta-actin was used as loading control. (C) Representative electron microscopy pictures (acid phosphatase staining) of Wt BMDM incubated with medium, cyclodextrin, or 27HC. Pictures of approximately 25 BMDM were scored for lysosomal cholesterol accumulation. Data were set relative to BMDM from Wt mice incubated with medium. *Indicates p <0.05; **p <0.01; ***p <0.001.

expression analysis for genes important for lysosomal cholesterol transport (*Npc1* and *Npc2*) was assessed. Whereas *Npc1* expression was not affected by LPS stimulation, the expression of *Npc2* increased dramatically after stimulation with LPS in both BMDM from Wt and *Cyp27a1*^{over} mice (Fig. 4A). Notably, macrophages overexpressing Cyp27a1 showed increased expression of *Npc2* compared to Wt BMDM after LPS stimulation (Fig. 4A). Additionally, in order to study specifically the effect of 27HC on macrophages, protein levels of *Npc1* and *Npc2* were determined in BMDM from Wt mice that were incubated with 27HC and cyclodextrin (carrier control). Interestingly, 27HC incubation led to a significant induction of NPC1 and NPC2 protein levels compared to control condition (Fig. 4B). This increase was accompanied by a reduction in lysosomal cholesterol accumulation in BMDM, incubated with 27HC as shown in the electron microscopy pictures (Fig. 4C). While adding cyclodextrin to macrophages resulted in a trend towards a reduction in lysosomal cholesterol accumulation, adding 27HC was able to dramatically reduce lysosomal cholesterol accumulation in comparison to control and compared to the cyclodextrin condition. Thus, 27HC is more effective in reducing lysosomal cholesterol accumulation than its vehicle cyclodextrin. Taken together, these data suggest that Cyp27a1 and 27HC are able to modulate intracellular cholesterol trafficking in macrophages via NPC proteins.

Discussion

Our results indicate that Cyp27a1 overexpression in hematopoietic cells reduces diet-induced hepatic inflammation. Remarkably, the reduction in hepatic inflammation was independent of plasma and liver levels of 27-hydroxycholesterol and other oxysterols. Mechanistically, our data suggest that Cyp27a1 can reduce hepatic inflammation via modulation of intracellular cholesterol trafficking in macrophages. Furthermore, our data provide further evidence to the importance of KCs in triggering hepatic inflammation.

CYP27A1 is able to reduce hepatic inflammation independent of circulating 27HC levels

Currently, most data are highly controversial regarding the activities of oxysterols. Although some *in vitro* studies have demonstrated that oxysterols have some cytotoxic, oxidative, and/or inflammatory effects [11], the most abundant oxysterol, being 27HC, is considered as a potential candidate for the reduction of inflammation during NASH [7]. Daily injection of 27HC was found to result in reduced hepatic inflammation in a dietary model for NASH. As expected, in addition to the reduced inflammatory response upon 27HC administration, increased levels of 27HC in liver and plasma were detected [7]. Importantly, 27HC was found to be a selective oestrogen receptor modulator that can serve as a competitive antagonist for the oestrogen receptor [12,13]. As such, circulating levels of 27HC levels may directly antagonize the functions of oestrogen receptors in vascular endothelial and smooth muscle cells, thereby leading to a loss of the cardioprotective effect of oestrogen [14]. Furthermore, increased levels of 27HC, which occur during hypercholesterolemia, have recently been shown to be involved in different pathologies. In a mouse model for breast cancer, it was shown that 27HC

increases oestrogen receptor-dependent growth and LXR-dependent metastasis [15]. Mice that demonstrated increased circulating levels of 27HC showed increased metastasis of breast cancer cells to the lung. Besides involvement in breast cancer, it was shown that increased concentrations of 27HC led to decreased bone mineral density that was associated with decreased bone formation and increased bone resorption [12]. Our current data indicate that increased Cyp27a1 expression, specifically in hematopoietic cells, does not alter circulating 27HC levels while inflammation is reduced. Our data suggest that the increased systemic and hepatic production of 27HC is prevented by upregulation of the classical pathway. Another explanation for the similar levels of systemic and hepatic levels of 27HC between the groups is the fact that parenchymal cells compromise about 80% of all liver cells. In our study, gene expression of *Cyp27a1* in total liver was not different between Wt-tp and *Cyp27a1*^{over}-tp mice. Therefore, hematopoietic overexpression of Cyp27a1 is not likely to be reflected in increased 27HC levels in plasma and liver. Therefore, our data further point towards therapy options wherein CYP27A1 is specifically targeted in KCs.

CYP27A1 modulates intracellular cholesterol trafficking via NPC proteins

The observation that hematopoietic Cyp27a1 overexpression leads to a reduced foamy appearance of KCs while neither cholesterol uptake, nor reverse cholesterol transport was modulated in total liver suggests that overexpression of Cyp27a1 only affected intracellular cholesterol trafficking inside KCs. Our current observations are in line with our previous findings that indicated that the agonistic effect of 27HC on the liver X receptor (LXR) in KCs is not dominant in all liver cells, but is restricted to KCs [7]. In line with our data it was shown that introducing the expression of Cyp27a1 *in vitro*, by transfecting cells, stimulated cholesterol efflux compared to untransfected control cells [16]. Additionally, pre-incubation of non-transfected cells with 27HC led to increased cholesterol efflux by 24–60% [16]. Others have reported that this process may occur via LXR-stimulation, as it is known that 27HC and possibly another product of CYP27A1, cholestenoic acid, may be ligands of LXR, regulating a number of genes involved in lipid metabolism including *Abca1* [17]. Interestingly, hepatic levels of cholesteryl esters were dramatically reduced in *Cyp27a1*^{over}-tp mice compared to the levels in Wt-tp mice in our study. In line with our findings, it was demonstrated that reduced cholesteryl esters, by transgenic overexpression of the cholesteryl ester hydrolase (CEH) in macrophages, polarizes Kupffer cells to a more anti-inflammatory phenotype that attenuates hepatic lipid synthesis and accumulation [18]. Furthermore, macrophage CEH overexpression was found to reduce atherosclerosis and necrosis in *Ldlr*$^{-/-}$ mice, while free cholesterol levels were unchanged [19,20]. In addition, accumulation of cholesteryl esters during cholesteryl ester storage disease is known to be associated with increased inflammation in different tissues and accelerated atherosclerosis [21].

Although 27HC was previously shown to be able to influence intracellular cholesterol transport from lysosomes to the cytoplasm [7,22], the mechanism involved is not yet known. The two main proteins that are involved in cholesterol transport from lysosomes to the cytoplasm are Niemann-Pick C1 (NPC1) and NPC2. While NPC1 is a multiple membrane spanning domain protein containing a sterol sensing domain, NPC2 is a small intralysosomal protein that has been characterized biochemically as a cholesterol-binding and transport protein [23]. A defect in either of these proteins results in Niemann-Pick disease type C, characterized by lysosomal cholesterol accumulation and inflammation in different tissues including the liver. Interestingly, it was shown that *NPC1* and *NPC2*-deficient cells have a severely reduced production of 27HC, and that upon incubation with 27HC the lysosomal cholesterol pool in *NPC1*$^{-/-}$ fibroblasts is dramatically reduced [22]. In line with these data, we observed for the first time that Cyp27a1 overexpression is able to increase NPC1 and NPC2 gene expression and protein levels. After binding, NPC2 is able to deliver cholesterol intracellular via interaction with the phospholipid bilayer, thereby reaching a putative transmembrane transporter via lateral diffusion in the plane of the membrane, or it could flip across the limiting lysosomal membrane and become accessible for transportation to the plasma membrane [24]. Alternatively, NPC2 is able to directly interact with NPC1 or other lysosomal membrane proteins, resulting in the removal of cholesterol from the lysosome [25]. In this way, NPC1 and NPC2 function as a tag team duo to mobilize cholesterol [24,26]. Our data suggest that 27HC can stimulate NPC-mediated cholesterol binding and transportation to the lysosomal membrane, where it can be released out of lysosomes. Taken together, our studies provide a new mechanism by which Cyp27a1 can modulate intracellular cholesterol trafficking in macrophages, thereby leading to reduced inflammation.

While both intracellular cholesterol trafficking and cholesterol efflux can modulate inflammation, the increased NPC expression observed in *Cyp27a1*^{over}-tp mice is probably the main reason for the reduced inflammatory response observed in *Cyp27a1*^{over}-tp mice. Increased cholesterol efflux was shown to modulate the immune response and inflammation through direct and indirect anti-inflammatory mechanisms [17]. However, it was reported previously that the total amount of cholesterol in cells is not correlated directly with inflammation, but rather the amount of cholesterol trapped inside lysosomes [2], suggesting an important role for NPC proteins in the inflammatory response. Moreover, studies in both arteries and in cell culture have shown that accumulated cholesterol in lysosomes cannot be decreased simply by inhibiting further uptake of lipoproteins or by increasing efflux of extra-lysosomal cholesterol stores [27–29]. On the other hand, increasing the expression of the NPC1 protein led to increased cholesterol efflux and the inhibition of atherosclerosis [30]. Thus, increased cholesterol efflux can also be a consequence of increased NPC expression. To conclude, while both pathways contributes to the reduced inflammatory response observed in the *Cyp27a1*^{over}-tp mice, stimulating cholesterol transport out of lysosomes seems to play a more dominant role.

In summary, we have shown for the first time that overexpression of Cyp27a1, specifically in macrophages, is able to reduce hepatic inflammation. Mechanistically, our data suggest that Cyp27a1 can modulate intracellular cholesterol trafficking by increasing NPC1 and NPC2 expression. This effect is likely regulated via increased intracellular levels of 27HC, while circulating levels of 27HC are unchanged between the transplanted groups. Taken together, our data point towards the potential of targeted therapy options during the development of NASH and other inflammatory-related disorders, such as atherosclerosis.

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Authors' contributions

TH, MLJJ, VB, SMAW, PJvG, TH, EL, RSS: study concept and design; TH, MLJJ, FV, YDG, MJJG, DL, RSS: acquisition of data; TH, MLJJ, PJvG, RSS: (statistical) analysis and interpretation of data; TH, MLJJ, RSS: drafting of the manuscript; TH, MLJJ, VB, SMAW, PJvG, FV, TH, YDG, MJJG, EL, MH, DL, RSS: critical revision of the manuscript; MH and RSS obtained funding.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2014.09.027.

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