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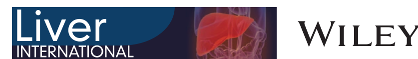

HDL cholesterol protects from liver injury in mice with intestinal specific LXRα activation

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Abstract

Background and aims: Liver X receptors (LXRs) exert anti-inflammatory effects even though their hepatic activation is associated with hypertriglyceridemia and hepatic steatosis. Selective induction of LXRs in the gut might provide protective signal(s) in the aberrant wound healing response that induces fibrosis during chronic liver injury, without hypertriglyceridemic and steatogenic effects.

Methods: Mice with intestinal constitutive LXRα activation (iVP16-LXRα) were exposed to intraperitoneal injection of carbon tetrachloride (CCl_4) for 8 weeks, and in vitro cell models were used to evaluate the beneficial effect of high-density lipoproteins (HDL).

Results: After CCl_4 treatment, the iVP16-LXRα phenotype showed reduced M1 macrophage infiltration, increased expression M2 macrophage markers, and lower expression of hepatic pro-inflammatory genes. This anti-inflammatory effect in the liver was also associated with decreased expression of hepatic oxidative stress genes and reduced expression of fibrosis markers. iVP16-LXRα exhibited increased reverse cholesterol transport in the gut by ABCA1 expression and consequent enhancement of the levels of circulating HDL and their receptor SRB1 in the liver. No hepatic steatosis development was observed in iVP16-LXRα. In vitro, HDL induced a shift from M1 to M2 phenotype of LPS-stimulated Kupffer cells, decreased TNFα-induced oxidative stress in hepatocytes and reduced NF-kB activity in both cells. SRB1 silencing reduced TNFα gene expression in LPS-stimulated KCs, and NOX-1 and IL-6 in HepG2.

Conclusions: Intestinal activation of LXRα modulates hepatic response to injury by increasing circulating HDL levels and SRB1 expression in the liver, thus suggesting this circuit as potential actionable pathway for therapy.

Abbreviations: ACAT2, Acetyl-Coa Acetyltransferase-2; ARG1, arginase 1; ECM, extracellular matrix; HDL, high-density lipoproteins; HO-1, heme oxygenase-1; HSCs, hepatic stellate cells (HSCs); IFN, interferon; IL, interleukin; KCs, Kupffer cells; LPS, lipopolysaccharide; LXR, Liver X receptors; MDA, malondialdehyde; MELD, Model for End-Stage Liver Disease; NF-kB, nuclear factor kappa-B; NOX, nicotinamide adenine dinucleotide phosphate oxidases; RCT, reverse cholesterol transport; ROS, reactive oxygen species; SRB1, scavenger receptor B type 1; SREBP-1c, sterol regulatory element-binding protein 1; TGFβ, transforming growth factor β; TIMP1, metallopeptidase inhibitor-1; TLR, Toll-like receptor; TNF, tumour necrosis factor; α-SMA, α-smooth muscle actin.

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KEYWORDS
fibrosis, high-density lipoproteins, inflammation, liver X receptors

1 | INTRODUCTION

Liver fibrosis is the results of the wound healing response in the course of chronic hepatic injury, independently from its etiology, and it is characterized by extracellular matrix (ECM) deposition and progressive alteration of the parenchymal architecture, leading to cirrhosis, portal hypertension and, finally, liver failure and hepatocellular carcinoma.[1] From 1999 to 2016 in the US annual deaths from cirrhosis increased by 65%, with people aged 25-34 years characterized by the greatest relative increase in mortality.[2]

In the classical pathological cascade, hepatic fibrosis is the final result of a complex interplay between damaged hepatocytes and reactive non-parenchymal liver cells, such as hepatic stellate cells (HSCs), Kupffer cells (KCs) and mononuclear cells recruited from peripheral blood, lymphocytes and endothelial cells. Among these cell populations, HSCs constitute the main effectors of liver fibrosis, contributing to about 90% of ECM synthesis and inflammatory signalling pathways which initiate and maintain liver damage.[1,3,4] Up to a few years ago, the research on hepatic fibrosis was mainly focused on the events occurring in liver resident cells, but recently it has expanded on the role of the so-called gut-liver axis because both dangerous and protective mechanisms for the liver can also derive from the intestine.[4,5]

Liver X receptors (LXRs) belong to the nuclear hormone receptor superfamily of ligand-activated transcription factors.[6] Two isoforms of LXRs have been described, respectively, LXRα and LXRβ, which share approximately the 78% of aminoacid sequences in their DNA and ligand-binding domain.[7] While LXRβ is found in almost all tissues, LXRα expression is restricted to specific tissues, such as liver, intestine, adipose tissue, kidney and macrophages.[7] LXRs are master regulators of whole-body cholesterol homeostasis via several mechanisms. One of these mechanisms is represented by the ability of LXRs to control the expression of members of the ATP-binding cassette transporters such as ABCG5 and ABCG8. These transporters are localized in the apical membrane of intestinal cells, where they limit cholesterol absorption from the gut, and in the hepatocytes, where they mediate cholesterol efflux into the bile.[8] Moreover, LXRs regulate cholesterol metabolism by triggering the reverse cholesterol transport (RCT). RCT occurs by the activation of ABCA1 and ABCG1 that next transfer excess of cholesterol from peripheral tissues to lipid-poor apoA-I and high-density lipoproteins (HDL), and finally to the liver, where via scavenger receptor B type 1 (SRB1) the HDL uptake into hepatocytes takes place.[8-10] HDL may exert anti-inflammatory and anti-oxidant effects in macrophages, neutralize circulating lipopolysaccharide (LPS) and modulate vascular resistance by reducing inflammation at the endothelial level in animal models as well as in patients with cirrhosis.[11-14] In patients with stable cirrhosis, baseline levels of HDL were significantly lower compared to controls predicting the development of liver-related complications independently of MELD.[15] Despite these lines of evidence, HDL role in chronic liver diseases has not been fully elucidated and the use of currently available pan-LXR agonists (that activate both isoforms), induces hepatic steatosis and hypertriglyceridemia by LXRα-mediated induction of sterol regulatory element-binding protein 1 (SREBP-1)c in hepatocytes.[4,16] Therefore, alternative strategies have been considered, especially in the field of atherosclerosis.[10] It has been demonstrated that the intestine can contribute through RCT to approximately 30% of the steady-state plasma HDL levels upon activation of the LXR target gene ABCA1.[17] Thus, pharmacological/genetic activation of intestinal LXR has been shown to reduce cholesterol overload by the induction of ABC transporters,[18,19] thus promoting cholesterol excretion from the gut, as well as, its efflux from vessel wall macrophages via HDL-mediated RCT.

The aim of the present study was to evaluate whether a selective intestinal activation of LXRα antagonize hepatic fibrosis onset through a protective gut-liver axis cross-talk.

Key points

Liver X receptors (LXRs) are master regulators of whole-body cholesterol homeostasis and also exert anti-inflammatory effects, but their hepatic activation is associated with the occurrence of steatosis and hypertriglyceridemia that can accelerate the progression of chronic liver injury.

In our study, selective intestinal LXRα activation lead to reverse cholesterol transport that protects from chronic liver injury progression by the anti-inflammatory effects of increased HDL cholesterol levels through the interaction with its receptor SRB1. The protective effect provided by the selective activation of LXRα in the gut was not associated with hepatic steatosis.

The selective pharmacological activation of intestinal LXRα and/or the increase of HDL cholesterol could represent a new therapeutic target of the gut-liver axis cross-talk to block the progression of chronic liver injury to hepatic fibrosis.

2 | MATERIALS AND METHODS

2.1 | In vivo model

Mice with constitutively activated form of LXRα in enterocytes, iVP16-LXRα and their specific controls iVP16, were kindly provided by Prof. Antonio Moschetta (University of Bari, Italy).[18] iVP16 or

iVP16-LXRα mice (from 5 to 8 weeks old) received a intraperitoneal injection of either carbon tetrachloride (CCl_4) (n = 10 for each group), at the dose of 1 μL/g of body weight, or corn oil (n = 5 for each group) twice a week for 8 weeks.[20] Mice were harvested 96 hours after the last dose of CCl_4. At the end of treatment, animals were anaesthetized and sacrificed according to institutional guidelines. All animal experiments were performed according to the guidelines of the Ancona University Institutional Animal Care and Use Committees.

2.2 | In vitro models

In order to study the effects of HDL in KCs we performed in vitro experiments using murine Kupffer cell lines (Abm® inc., Vancouver, CND). KCs were grown in Prigrow II (Abm® inc., Vancouver, CND) medium at 37°C in a humidified 5% CO_2 atmosphere. Cells were plated at 70% of confluence and after 12 hours were exposed to LPS (200 ng/mL) for 1 hours with or without a 16 hours pre-incubation (O/N) with either 1 mg/mL or 2 mg/mL HDL (Sigma-Aldrich, Saint Louis, MO).[12]

The effects of HDLs have also been evaluated in HepG2 cells (liver hepatocellular carcinoma cells, LGC Standards Srl., Italy) that were grown in Minimum Essential Medium (MEM). Cells were incubated with 100 ng/mL tumour necrosis factor (TNF)γ (Sino Biological Inc, Beijing, PRC) for 6 hours with or without a 16 hours pre-incubation (O/N) with either 1 mg/mL or 2 mg/mL HDL (Sigma-Aldrich, Saint Louis, MO).

Immunofluorescence was performed on cells plated on chamber slides and fixed in ice-cold methanol/acetone (ratio 3:1) for 10 minutes at −20°C. Cells were next incubated with primary antibody (mouse monoclonal anti-p65NF-kB antibody, 1:80, Santa Cruz Biotech.) for 2 hours at room temperature in PBS-BSA. Cells were washed three times in PBS-BSA before addition of the appropriate Alexa 488-conjugated anti-species IgG diluted to 1:500 in PBS-BSA for 1 hour at room temperature. Nuclei were counterstained with Hoechst. The images were acquired using the Olympus Fluoview FV1000 confocal microscope. The average intensity of p65NF-kB into the nucleus of each cell was measured by ImageJ software for at least 10 fields per condition of treatment.

Finally, we analysed the effects of HDL in murine macrophages. At this purpose, Raw 264.7 cells (American Type Culture Collection, USA) were maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich, Milan, Italy) and incubated with LPS (200 ng/mL for 1h) and/or IFNγ (20 ng/mL for 3 hours) both chemicals purchased by Sigma-Aldrich (Milan, Italy) with or without O/N pre-incubation with HDL.

To evaluate the role of the HDL cholesterol receptor SRB1, silencing experiments were performed in KCs and HepG2 cells 25×10^4 cells were grown in 6-well plates. The KCs and HepG2 cells were transfected 24 hours after plating using, respectively, 2 μL siPORT neoFX (ThermoFisher Scientific Baltics UAB) and 4 μL INTERFERin® (Polyplus-transfection S.A, Illkirch, France), according to manufacturer's instruction either with selected predesigned siRNA or nt-RNA (Ambion Life Technologies Corporation, Woburn, MA). The KCs were then exposed to LPS (200 ng/mL) for 1 hour with or without a 16 hours preincubation (O/N) with 2 mg/mL HDL (Sigma-Aldrich, Saint Louis, MO), while the HepG2 cells were then exposed to TNFα (100 ng/mL) for 6 hours with or without a 16 hours pre-incubation (O/N) with 2 mg/mL HDL (Sigma-Aldrich, Saint Louis, MO). The effect of SRB1 silencing was evaluated by measuring mRNA expression of TNFα, NOX1, IL-6 and HO-1.

2.3 | Experimental procedures

Detailed descriptions of experimental procedures are provided in Data S1.

3 | RESULTS

3.1 | Intestinal activation of LXRα reduces hepatic inflammation in CCl_4-treated mice

The hepatotoxin CCl_4 is known to induce hepatic injury, including hepatocytes necrosis, inflammation and fibrosis.[20] Thus, iVP16 and iVP16-LXRα were exposed to intraperitoneal injection of CCl_4 for 8 weeks, in order to analyse the effect of intestinal LXRα activation on chronic liver damage.

During liver injury progression, activation of hepatic macrophages represents a major source of inflammatory mediators including cytokines, eicosanoids and chemokines which are stimuli sustaining HSCs activation and fibrogenesis.[3] Thus, we performed immunohistochemistry for the macrophage marker F4/80 and we observed that CCl_4- induced cell infiltration was significantly decreased in iVP16-LXRα mice compared to controls (Figure 1A, Figure S1A). Moreover, in the liver of iVP16-CCl_4 we observed a dramatic reduction in the histological number of CD68+/CD206 + M2 macrophages (Figure 1B) coupled to a significant decrease of the hepatic mRNA and protein expression of the M2 macrophage marker Arginase 1 (ARG1) (Figure 1C, Figure S1B) and interleukin (IL)-10 (Figure 1D). When compared to iVP16-CCl_4, iVP16-LXRα CCl_4-treated mice showed a recovery of M2 macrophages analysed by CD68/CD206 ratio, and ARG1 and IL-10 gene expression (Figure 1B-D). Data on the anti-inflammatory activity of intestinal LXRα activation were also confirmed by the analysis of p65NF-kB, one of the five components that form the NF-kB transcription factor family involved in the inflammatory response.[21] As shown in Figure S1C, nuclear translocation increased in iVP16-α CCl_4-treated mice and was down to control level in iVP16-LXRα CCl_4-treated mice.

Hepatic inflammation reduction in iVP16-LXRα CCl_4-treated mice vs iVP16 CCl_4-treated mice was also supported by down-regulation of the well-known pro-inflammatory LPS-toll-like receptor (TLR)4 signalling pathway.[22,23] In particular, we found a significant decreased expression of TLR4, NF-kB, IL-6, TNFα and IFNβ genes (Figure 1E).

These results thus suggest that LXRα activation in the intestine can modulate liver inflammation, a critical step in the development of chronic hepatic injury and fibrosis.

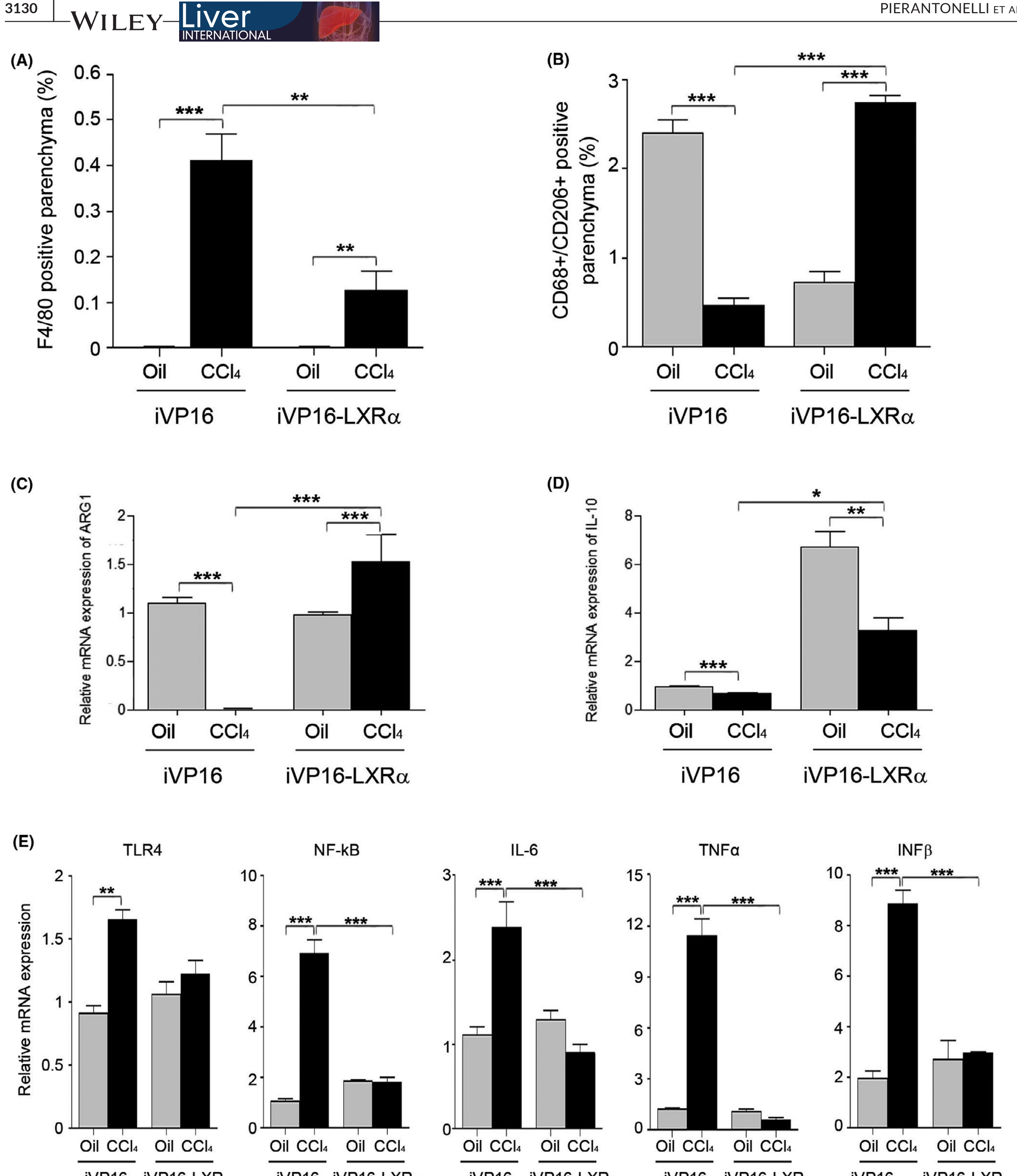

FIGURE 1 Inflammatory markers in the liver of iVP16 and iVP16-LXRα mice treated with oil or CCl_4. (A) Immunohistochemistry for the macrophage marker F4/80. (B) Quantitative analysis of the number of M2 macrophages (CD68/CD206 positive). Relative mRNA expression by qRT-PCR for ARG1 (C), IL-10 (D), and TLR4, NF-kB, IL-6, TNFα and IFNβ. Mean ± SD. **P* < .05; ***P* < .01; ****P* < .001. All experiments were performed in triplicate

3.2 | Intestinal LXRα activation reduces hepatic oxidative stress in CCl_4-treated mice

A well-known mechanisms by which chronic liver inflammation promotes hepatic fibrosis is represented by the occurrence of oxidative stress, characterized by excessive reactive oxygen species (ROS) formation.[4]

As expected, we found an increase in malonildialdehyde (MDA) hepatic levels in iVP16CCl_4-treated mice that was inhibited in iVP16-LXRα CCl_4-treated mice (Figure 2A).

Emerging evidences indicate that the nicotinamide adenine dinucleotide phosphate oxidases (NOXs) are sources of ROS, which play crucial roles in the progression of hepatic fibrosis.[24] Thus, we evaluated the expression levels of NOX1, highly expressed in hepatocytes as well as in HSCs, and NOX2, that plays an important role in inflammation, host immune defense and oxidative stress in KCs.[25] iVP16 CCl_4-treated mice exhibited a significant increase of both NOX1 and NOX2 gene/protein expression that was hindered by intestinal LXRα (Figure 2B,C, Figures S2A,B).

The increased oxidative stress was also associated with a dramatic decrease in the expression of heme oxygenase-1 (HO-1), an enzyme involved in anti-inflammatory and anti-fibrotic processes,[26] in iVP16 CCl_4-treated iVP16 mice. While in iVP16- LXRα CCl_4-treated mice HO-1 levels were similar to those observed in controls (Figure 2D).

These results suggest that intestinal LXRα activation in the gut decreases hepatic oxidative stress induced by CCl_4 treatment.

3.3 | Intestinal LXRα activation reduces hepatic fibrosis during chronic liver injury

No evidence of histological hepatic fibrosis was observed in control mice. The degree of hepatic fibrosis was graded according to the Metavir score and was 2.18 ± 0.5 in iVP16- CCl_4 mice vs 0.6 ± 0.49 in iVP16-LXRα CCl_4-treated mice ($P < .001$). In order, to investigate whether suppression of hepatic fibrosis was associated with decreased fibrogenesis, we firstly assessed gene and protein expression of α-smooth muscle actin (α-SMA), a marker of HSCs activation.[27] As shown in Figure 3A, the increased mRNA expression of α-SMA induced by CCl_4 in iVP16 mice was significantly reduced in iVP16-LXRα mice and this result was confirmed by immunohistochemistry (Figure 3B, Figure S3A). After CCl_4 treatment and compared to iVP16 animals, decreased HSCs activation in iVP16-LXRα was associated with reduced mRNA levels of TGFβ, TIMP1 and COL1A1, as key elements involved in the fibrogenetic process (Figure 3C-E). Overall this resulted in a lower collagen deposition detected in iVP16-LXRα mice after CCl_4 treatment compared to iVP16 CCl_4-treatd mice, as evaluated by both measurement and morphometry for Sirius Red positive staining and hydroxyproline content (Figure 3F, Figures S3B,C).

Taken together these data indicate that protection provided by intestinal activation of LXRα in terms of reduced inflammation and oxidative stress finally results in reduced fibrosis.

3.4 | Reverse cholesterol transport associates with increased HDL levels and hepatic SRB1 expression

The protective role of the selective intestinal LXRα activation could be RCT. Indeed, the intestine represents, after the liver, the main organ involved in RCT and it has been shown that enterocytes account for 30% of HDL production.[17]

We found that intestinal expression of ABCA1 gene was increased in iVP16-LXRα mice, independent of CCl4 treatment

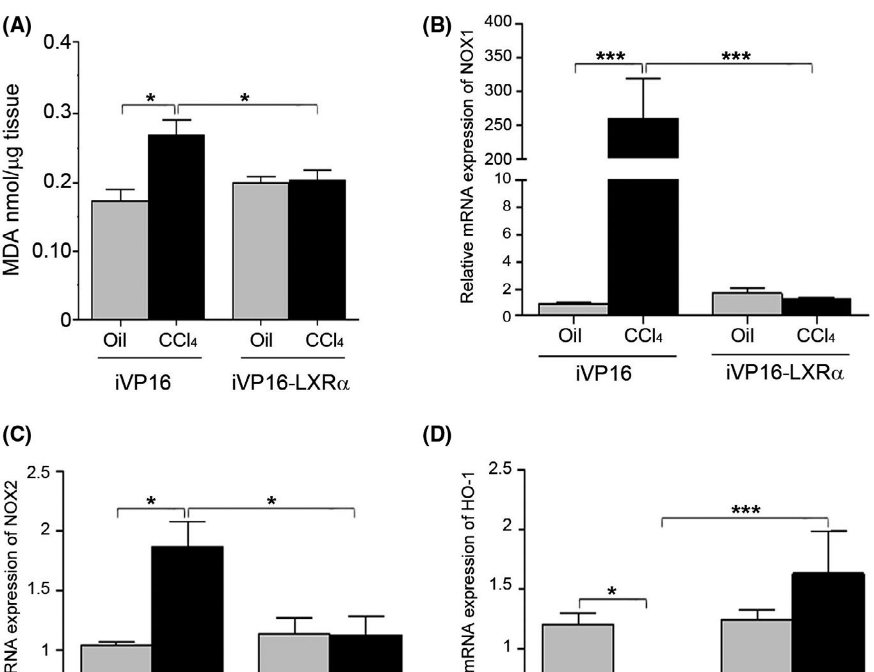

FIGURE 2 Oxidative stress in the liver of iVP16 and iVP16-LXRα mice treated with oil or CCl_4. (A) Quantitative analysis of MDA levels. Relative mRNA expression by qRT-PCR for NOX1 (B), NOX2 (C), and HO-1 (D). Mean ± SD. *$P < .05$; **$P < .01$; ***$P < .001$. All experiments were performed in triplicate

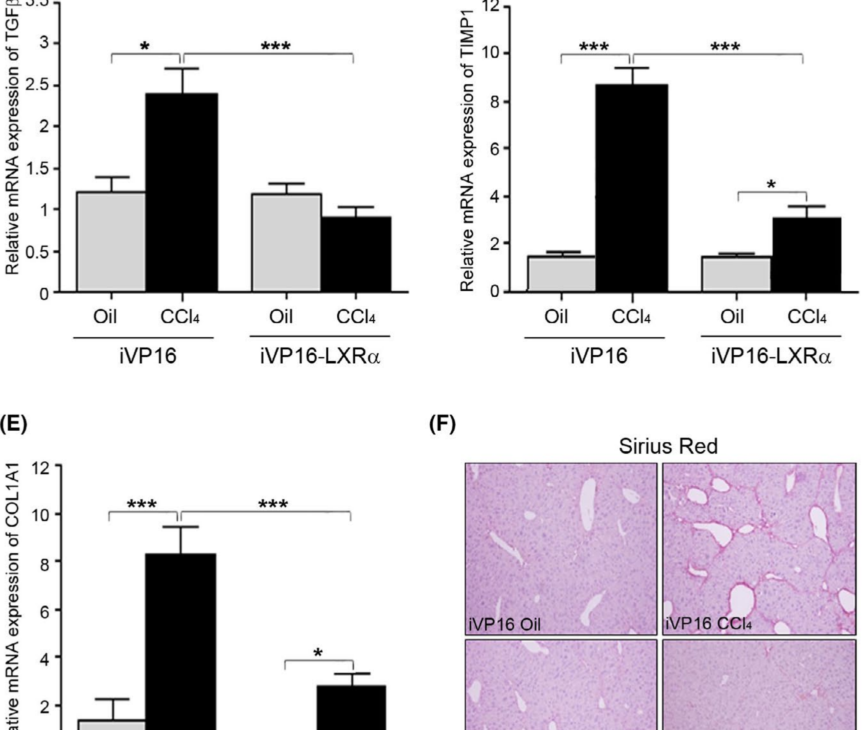

FIGURE 3 Hepatic fibrogenesis and collagen deposition in iVP16 and iVP16-LXRα mice treated with oil or CCl_4. (A) Relative mRNA expression by qRT-PCR for α-SMA. (B) Representative staining of the profibrogenetic marker α-SMA. Relative mRNA expression by qRT-PCR for TGFβ (C); TIMP1 (D), and COL1A1 (E). (F) Representative picture of Sirius Red staining. Mean ± SD. $^*P < .05$; $^{**}P < .01$; $^{***}P < .001$. All experiments were performed in triplicate

(Figure 4A). Conversely, CCl4 caused a reduction of ABCA1 hepatic gene expression, even if in the presence of a selective activation of LXRα this reduction is of minor entity (Figure 4B).

The net effect of increased RCT in iVP16-LXRα mice was then assessed by measuring lipoprotein levels in plasma. As shown in Figure 4C, iVP16-LXRα mice showed higher total cholesterol levels compared with iVP16 mice, either in oil or in CCl_4-treated mice. CCl_4 treatment caused a decrease of plasma HDL levels, but this decrease was mitigated by intestinal LXRα activation (Figure 4D).

Finally, an increased hepatic expression of the HDL cholesterol receptor SRB1 was observed in iVP16-LXRα CCl_4-treated mice compared to iVP16 CCl_4-treated mice (Figure 4E). Interestingly, as shown in Figure 4F, SRB1 is expressed in all liver-resident cells, including CD31 positive endothelial cells.

Thus, all of these findings suggest that RCT occurs in mice with a selective intestinal LXRα where it is associated with increased HDL circulating levels and SRB1 expression.

3.5 | Intestinal LXRα activation induces its target genes only in gut without steatogenic effects on the liver

The major side effects of LXR systemic activation are represented by liver steatosis and hypertriglyceridemia, because of LXRα induction of SREBP-1c in hepatocytes, which enhances lipogenesis.[10]

In light of these concerns, LXRα target genes were then measured in the gut as well as in the liver. The expression of the LXRα

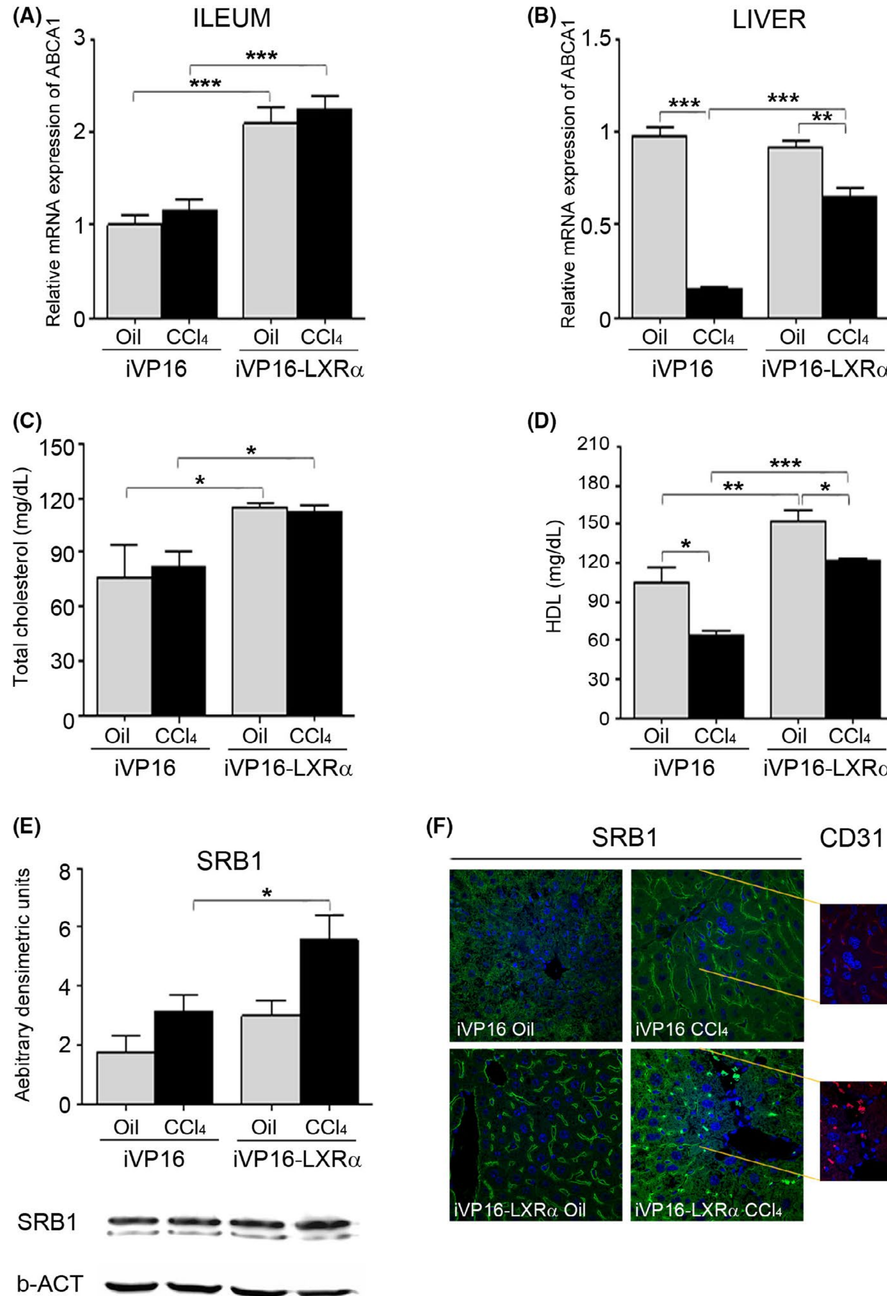

FIGURE 4 RCT in iVP16 and iVP16-LXRα mice treated with oil or CCl_4. Relative mRNA expression by qRT-PCR for ABCA1 in ileum (A) and liver (B). Plasma total cholesterol (C) and HDL cholesterol (D). (E) Quantitative analysis and representative image of hepatic expression of SRB1 protein. (F) Representative fluorescent image of the hepatic expression of SRB1 (green) and CD31 (red). Mean ± SD: $^*P < .05$; $^{**}P < .01$; $^{***}P < .001$. All experiments were performed in triplicate

target genes ABCG5 and ABCG8, involved in cholesterol transport, was increased in the intestine of iVP16-LXRα mice, either oil- or CCl_4-treated, compared to iVP16 (Figure 5A,B). On the contrary, the expression of same genes was unaffected in the liver (Figure 5C-D). Accordingly, the selective activation of LXRα only in gut niche has no effect on liver steatosis (Figure 5E), the lipogenic SREBP-1c (Figure 5F) and hepatic triglycerides content (Figure 5G).

Taken together these results indicate that iVP16-LXRα mice exhibit a specific intestinal LXR activation that protects from chronic liver injury progression without the occurrence of side effects (ie hepatic triglyceride accumulation) related to systemic LXRs activation.

3.6 | HDL directly polarizes macrophages towards the anti-inflammatory state

Our data indicate that iVP16-LXRα mice show reduced inflammation and oxidative stress, thus leading to reduced extracellular matrix deposition. These effects were not associated to increased LXR activity in the liver as shown by the lack of modifications of the LXR downstream effectors ABCG5 and ABCG8. On the other hand, the antifibrotic effect was associated with increased HDL circulating levels and SRB1 expression in the liver. Thus, to explain the mechanism leading to reduced liver injury in iVP16-LXRα CCl_4-treated mice, we hypothesized a role of HDL in reducing macrophages-mediated inflammation and oxidative stress, and consequently liver fibrosis,

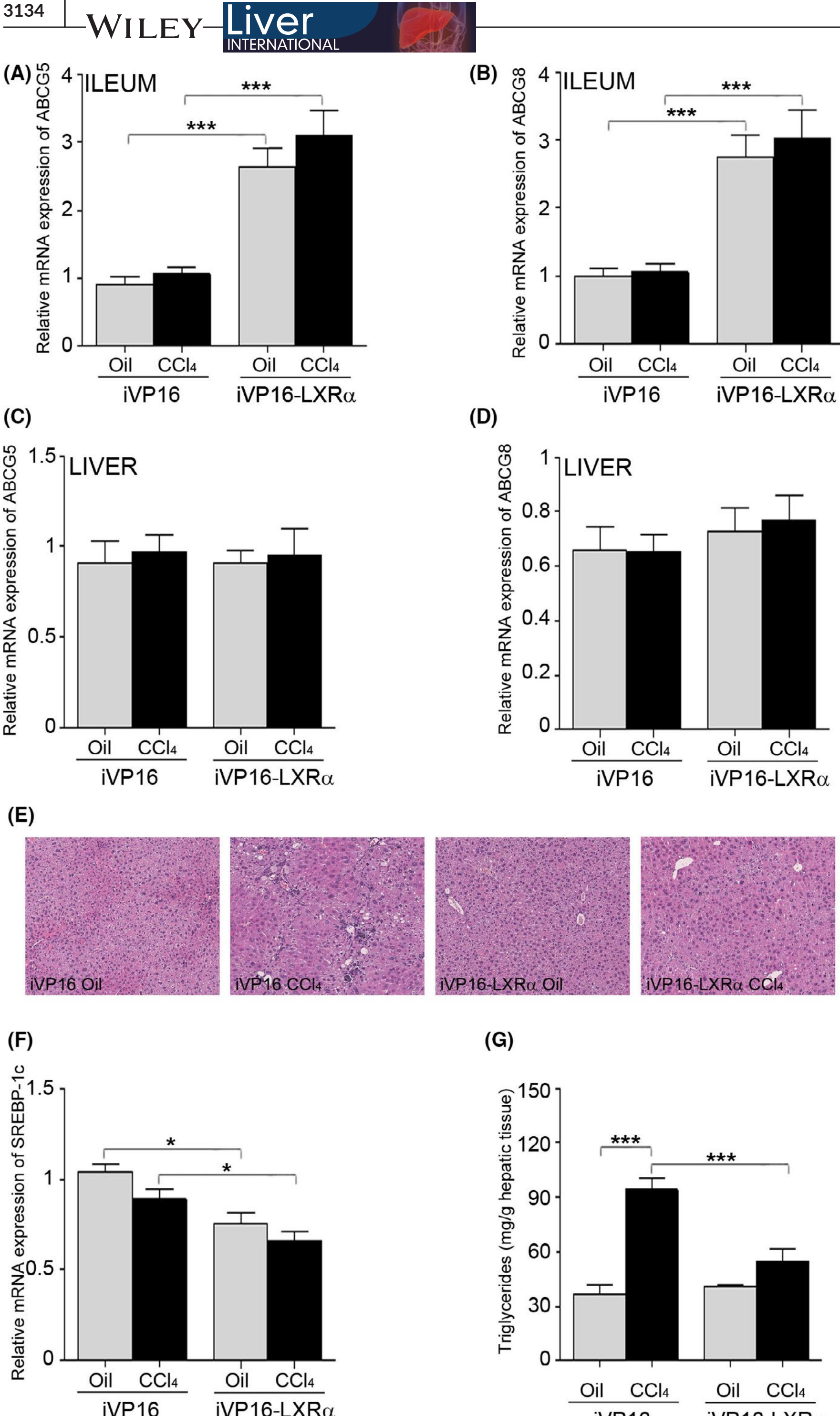

FIGURE 5 Steatogenic effect of selective activation of the intestinal LXRα in iVP16 and iVP16-LXRα mice treated with oil or CCl_4. Relative mRNA expression by qRT-PCR for ABCG5 (A) and ABCG8 (B) genes in the ileum. Relative mRNA expression by qRT-PCR for ABCG5 (C) and ABCG8 (D) genes in the liver. (E) Representative images of haematoxylin and eosin staining. (F) Relative mRNA expression by qRT-PCR for hepatic SREBP-1c. (G) Hepatic triglyceride content measured by colorimetric assay. Mean ± SD: $^*P < .05$; $^{***}P < .001$. All experiments were performed in triplicate

since iVP16LXRα showed reduced expression of NOX2, thus indicating a major pathogenetic role of KCs.[25]

It has been reported that CCl_4 damage is, at least in part, mediated by LPS effect in the liver.[28] Thus, KCs were primed in culture by LPS incubation to evaluate the eventual protective effect of HDL. HDL preincubation significantly reduced p65NF-kB activity (ie nuclear localization) induced in KCs by LPS (Figure S4A,B). HDL preincubation, also in the presence of LPS, increased the M2 macrophage marker expression ARG1 at either 1 or 2 mg/mL (Figure 6A), thus mimicking what has been observed in vivo. Moreover, HDL pre-incubation increased HO-1 expression in KCs, at both concentrations, in the presence of LPS (Figure 6B). On the other hand, LPS incubation increased the expression of the M1 phenotype markers IL1-β, IL-6 and TNFα (Figure 6C-E). The increased level of pro-inflammatory markers was abrogated by KCs preincubation with HDL, at either 1 mg/mL or at 2 mg/mL (Figure 6C-E).

Finally, taking advantage of the in vivo observation concerning increased SRB1 expression in mice protected from hepatic fibrosis, we evaluated the role of this receptor in mediating the protective effect of HDL in KCs by transient silencing approach with an efficiency

of 60% SRB1 reduction (Figure S5). In Figure 6F, HDL reduced the increased TNFα expression induced in KCs by LPS, while this effect was lost in KCs with reduced SRB1 expression.

The effect of HDL on inflammatory markers was also further analysed in a second cell line, Raw 264.7 cells, of peripheral murine macrophages. Raw 264.7 cells were activated by using LPS and/or IFNγ with or without HDL preincubation. As shown in Figure S6A-D, HDL preincubation again reduced IL-1β, IL-6, and TNFα, and increased HO-1 gene expression.

3.7 | HDL decreases oxidative stress in hepatocytes

We hypothesized that the increased levels of TNFα gene expression in the liver of iVP16-LXRα CCl_4-treated mice, known to be produced by activated KCs, could act in a paracrine mechanism on hepatocytes.[29] Indeed, in vitro experiments showed that TNFα treatment increased NOX1 and p65NF-kB gene expression (Figure 7A,B) and p65NF-kB nuclear activity (Figure S7A,B) in HepG2 cells. These increases were significantly reduced by HDL preincubation (Figure 7A,B and Figure S7A,B). In HepG2 cells, as well as in KCs, HDL preincubation caused a strong increase of HO-1 gene expression also in the presence of LPS (Figure 7C).

Transient silencing of SRB1 (Figure S8) resulted in the impairment of the protective effect of HDL in HepG2 cells treated with TNFα by increasing the expression of NOX1 and IL-6 genes (Figure 7D,E), and reducing OH-1 gene expression (Figure 7F).

4 | DISCUSSION

Liver fibrosis results from a complex interplay between parenchymal and non-parenchymal cells when pathological stimuli trigger hepatocyte damage and KC activation, which in turn causes the release of pro-inflammatory and pro-oxidant molecules responsible for collagen deposition.[3] Several pathogenetic aspects remain to be elucidated and unfortunately nowadays no specific treatments have been approved for this pathological condition.

In the present study, we have demonstrated that the specific activation of LXRα in the gut counteracted the progression of hepatic injury in the classical CCl_4 mouse model of liver fibrosis through the

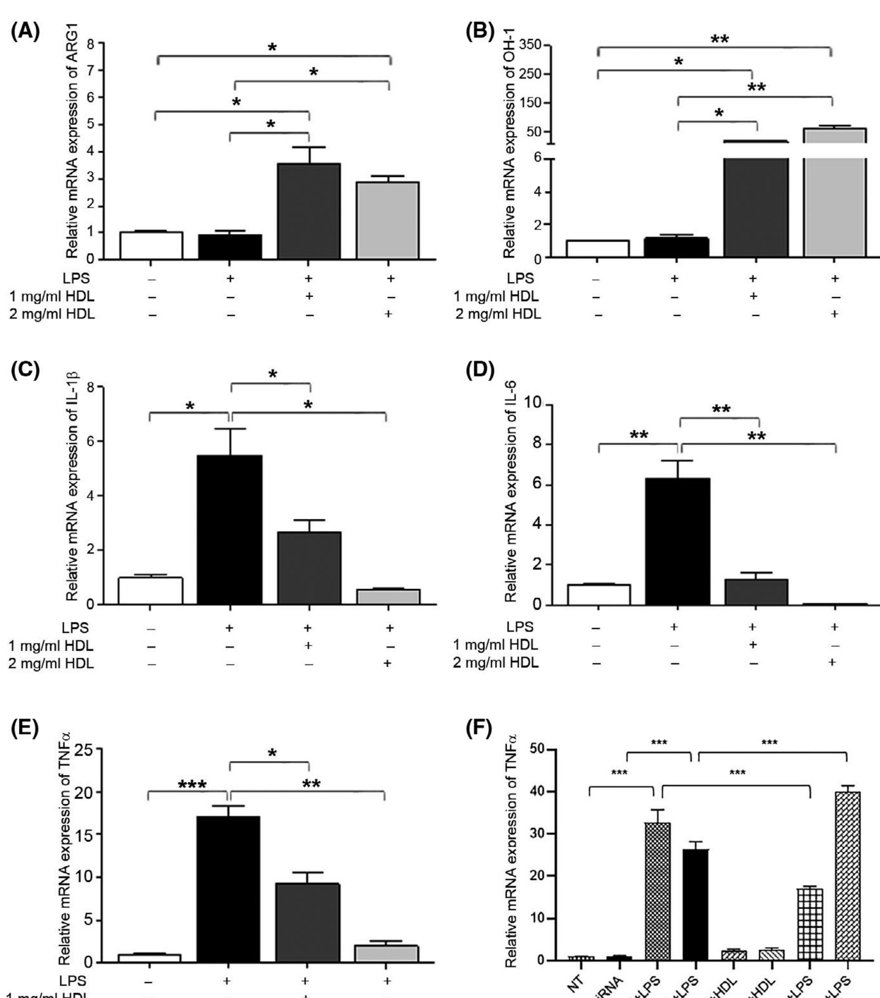

FIGURE 6 Effects of HDL on KCs in vitro. Relative mRNA expression by qRT-PCR for ARG1 (A), HO-1 (B), IL-1β (C), IL-6 (D) and TNFα (E) in LPS-activated KCs in the presence or not of HDL pre-incubation. (F) Relative mRNA expression by qRT-PCR for TNFα in KCs silenced for SRB1 or scramble siRNA treated with HDL or LPS alone or in combination. Mean ± SD: *$P < .05$; **$P < .01$; ***$P < .001$. All experiments were performed in triplicate of two independent experiments

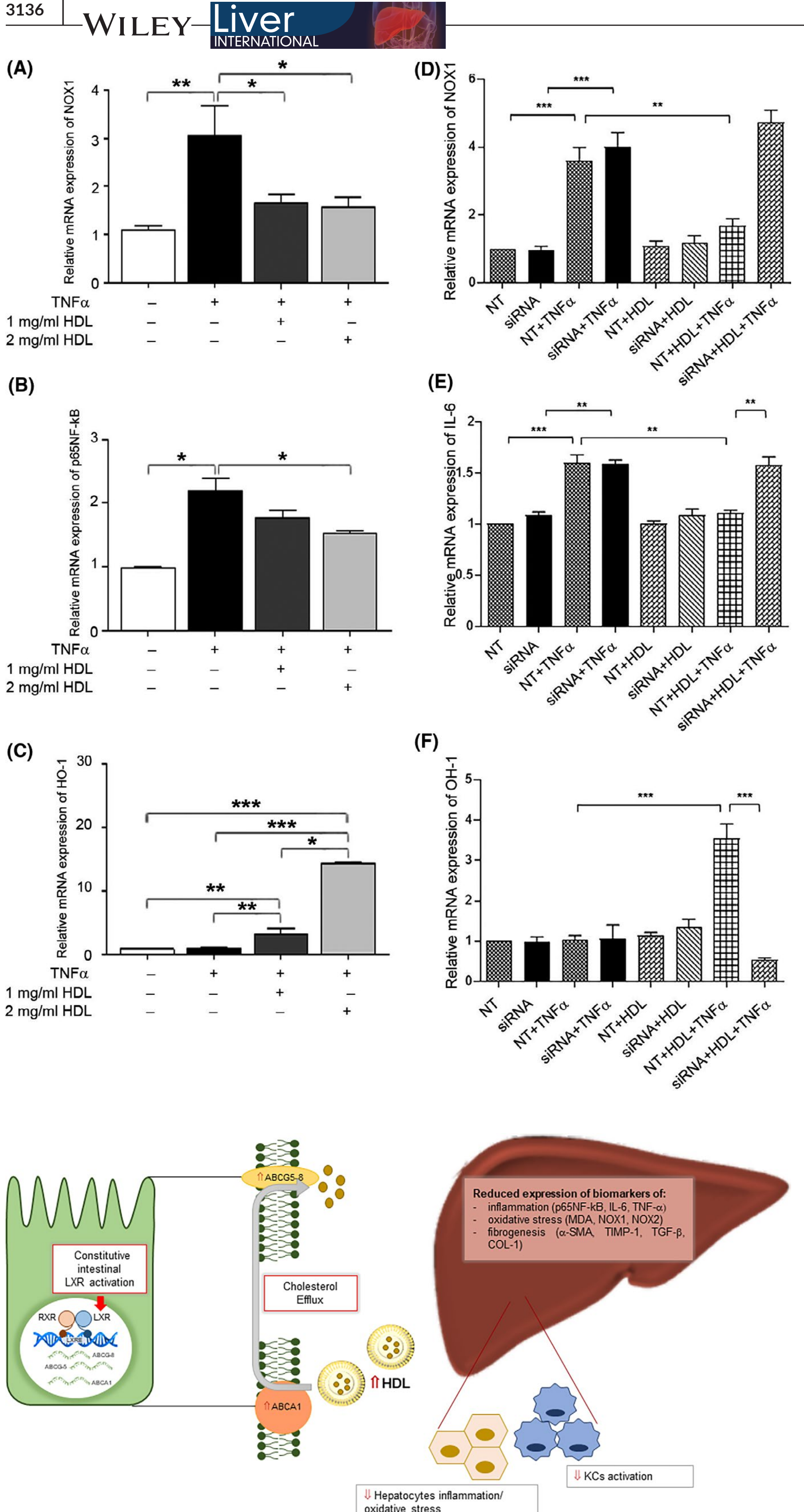

FIGURE 7 Effects of HDL on hepatocytes in vitro. Relative mRNA expression by qRT-PCR for NOX1 (A), p65NF-kB (B), and HO-1 (C) in TNFα-treated HepG2 cells in the presence or not of HDL pre-incubation. Relative mRNA expression by qRT-PCR for NOX1 (D), IL-6 (E), and HO-1 (F), in HepG2 cells silenced for SRB1 or scramble siRNA treated with HDL or TNFα alone or in combination. Mean ± SD: $^*P < .05$; $^{**}P < .01$; $^{***}P < .001$. All experiments were performed in triplicate of two independent experiments

FIGURE 8 Schematic representation of the protective mechanism of intestinal LXR activation on the progression of chronic liver injury

anti-inflammatory and anti-oxidant activity exerted by HDL cholesterol and the interaction with its receptor SRB1 (Figure 8). These results were also corroborated by in vitro studies on both KCs and hepatocytes.

We found that intestinal LXRα activation decreased liver injury in the classical mouse model of liver fibrosis induced by intraperitoneal injection of CCl_4, as shown by reduced hepatic inflammation evaluated by histological score, macrophage infiltration, p65NF-kB

nuclear translocation, and pro-inflammatory cytokines and oxidative stress genes expression. Furthermore, we found that LXRα activation in the gut induced a shift in the macrophage phenotype from the pro-inflammatory M1 to the anti-inflammatory M2, as demonstrated by the increased number of CD68/CD206 positive cells and levels of ARG1 and IL-10 gene expression in the liver.[30] The lowering of inflammatory activity and the reduction of oxidative stress explain the decrease in hepatic fibrogenesis and collagen deposition in CCl_4-treated mice with intestinal LXRα activation.

Importantly, in our study, no signs of increased LXRs activity have been found in the liver, as shown by the lack of increased hepatic expression of LXRs downstream targets ABCG5, ABCG8, ABCA1 and SREBP-1c. Otherwise, it has been demonstrated that LXR activation in the intestine can provide favourable effects through RCT that increases by almost 30% the steady-state plasma HDL levels upon activation of the LXR target gene ABCA1.[17-19] In iVP16-LXRα mice, either oil- or CCl_4-treated, activation of RCT as intestinal source of HDL was demonstrated by the increased ileal expression of the cholesterol transporter ABCA1 (that is involved in HDL formation), and this was coupled with increased circulating HDL levels.[18] In our model the anti-fibrotic effect in iVP16-LXRα mice was thus associated not only with a striking increase in circulating HDL levels, but also with higher hepatic expression of the HDL receptor SRB1 in all liver resident cells, including liver sinusoidal endothelial cells. On the contrary, decreased production of HDL occurs in cirrhotic patients, mainly attributed to a decreased synthetic capacity of the liver, and this has been associated with a pro-inflammatory status.[14] This has been confirmed in our model where the dramatic reduction in ABCA1 expression in the liver of CCl_4-treated iVP16 mice strongly confirms the lack of hepatic LXRs activation and reflects the reduced HDL production occurring during liver fibrosis. More recently it has been also shown that baseline levels of HDL were significantly lower in patients with stable cirrhosis than in healthy subjects, and this predicted the development of liver-related complications independently of MELD score.[15] In experimental models, as well as in patients with severe cirrhosis, recombinant HDL infusion was able to reduce concentration of pro-inflammatory cytokines by neutralizing circulating LPS.[13,14]

In order to investigate the mechanism(s) underlying the protective role exerted by HDL in iVP16-LXRα mice in vivo, we performed in vitro experiments by using cell lines of the two main liver cell phenotype involved in the release of pro-fibrogenic signals, ie KCs and hepatocyte,,[1,3,4] although a role of LSEC can also be hypothesized.[31] A major role of LPS-stimulated KCs in our model of fibrosis can derive both from the literature[28] and from the observation that NOX2, mainly expressed in hepatic phagocytic cells such as KCs and macrophages recruited from peripheral monocytes,[32] increased its expression in vivo. Either peripheral or resident macrophages, such as KCs, express specific receptors able to bind HDL particles.[33] Mimicking the in vivo situation, in vitro HDL pretreatment of KCs reverted the pro-inflammatory phenotype and: a) increased gene expression of ARG1, a well-known M2 marker[30]; b) increased HO-1 gene expression, as a defensive mechanisms against oxidative stress[26]; c) blocked NF-kB activity assessed by nuclear translocation. Similar results were obtained in peripheral murine macrophages stimulated with LPS and/or IFNγ.

We also verified whether the anti-inflammatory and anti-fibrotic role of HDL in iVP16-LXRα mice could also derive from an effect on hepatocytes, a cell population that also expresses HDL receptors,[33] by using the HepG2 cells and hypothesizing that TNFα released by activated hepatic macrophages could act in a paracrine manner on hepatocytes.[1,3] Similarly to what was observed in vivo, HDL was able to reduce both markers of inflammation and oxidative stress, and this was associated with reduced NF-kB nuclear translocation and increased HO-1 expression that represents a protective response by reducing both inflammation and fibrogenesis.[26,34]

SRB1 is a multiligand membrane receptor encoded by the gene Scarb1 that functions as a physiologically relevant HDL receptor. SRB1 in the liver, facilitates the selective delivery of HDL to hepatocytes. Furthermore, when binding to its ligand HDL, SRB1 regulates peripheral macrophages inflammation through activation of Akt, reduced activation of NF-κB and increased anti-inflammatory cytokines production.[35] We observed increased SRB1 levels in iVP16-LXRα CCl_4-treated mice protected from fibrosis, together with reduced p65-NF-kB activity. Moreover, transient silencing of SRB1 blocked the protective effects of HDL on LPS-induced KCs and TNFα-treated HepG2 cells, thus suggesting that HDL exert its action via SRB1.

Our findings suggest that approaches able to raise the concentration of HDL lipoprotein might be effective in counteracting the progression of chronic liver diseases. Unfortunately, the use of LXR ligands, which enhance HDL synthesis and regulate cholesterol homeostasis, is not practicable because systemic activation of LXRs may cause side effects because of LXRα induction in hepatocytes and consequent hepatic steatosis and hypertriglyceridemia. Accordingly to previous evidence,[18,35,36] our data confirms that the specific intestinal activation of LXRα could be an actionable pathway without modifying hepatic lipid metabolism.[37]

In conclusion, we showed that intestinal LXRα activation increases HDL levels and exerts anti-inflammatory and anti-oxidant effects on hepatic macrophages and parenchymal cells, finally ameliorating hepatic response to chronic injury (Figure 8). Hence, approaches that point to increase the HDL production, via intestinal LXRα activation, may represent new strategies for the treatment of chronic liver diseases.

5 | Financial information

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTION

IP, GL, FG, AA and GSB conceived and designed the experiments; IP, GL, FG, DMG, CR, CB, LT, EN, NP, CDS, VD, LM performed research; IP, GL, FG, NP, MM, MP, AA and GSB performed data interpretation; IP, GL, FG, MM, MP, AA, and GSB wrote the first-draft of the manuscript; all of the authors revised and approved the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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