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Research Article

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Obeticholic acid reduces bacterial translocation and inhibits intestinal inflammation in cirrhotic rats

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Background & Aims: In advanced cirrhosis, gut bacterial translocation is the consequence of intestinal barrier disruption and leads to bacterial infection. Bile acid abnormalities in cirrhosis could play a role in the integrity of the intestinal barrier and the control of microbiota, mainly through the farnesoid X receptor. We investigated the long-term effects of the farnesoid X receptor agonist, obeticholic acid, on gut bacterial translocation, intestinal microbiota composition, barrier integrity and inflammation in rats with CCl_4-induced cirrhosis with ascites.

Methods: Cirrhotic rats received a 2-week course of obeticholic acid or vehicle starting once ascites developed. We then determined: bacterial translocation by mesenteric lymph node culture, ileum expression of antimicrobial peptides and tight junction proteins by qPCR, fecal albumin loss, enteric bacterial load and microbiota composition by qPCR and pyrosequencing of ileum mucosa-attached contents, and intestinal inflammation by cytometry of the inflammatory infiltrate.

Results: Obeticholic acid reduced bacterial translocation from 78.3% to 33.3% (p <0.01) and upregulated the expression of the farnesoid X receptor-associated gene small heterodimer partner. Treatment improved ileum expression of antimicrobial peptides, angiogenin-1 and alpha-5-defensin, tight junction proteins zonulin-1 and occludin, and reduced fecal albumin loss and liver fibrosis. Enteric bacterial load normalized, and the distinctive mucosal microbiota of cirrhosis was reduced. Gut immune cell infiltration was reduced and inflammatory cytokine and Toll-like receptor 4 expression normalized.

Conclusions: In ascitic cirrhotic rats, obeticholic acid reduces gut bacterial translocation via several complementary mechanisms at the intestinal level. This agent could be used as an alternative to antibiotics to prevent bacterial infection in cirrhosis.

Introduction

The translocation of bacteria and bacterial products from the gut (GBT) is a hallmark of spontaneous bacterial infection, the systemic inflammatory response and remote organ injury in cirrhosis [1,2]. The high rate of GBT observed in patients and animal models of cirrhosis and ascites is the consequence of concurrent damage at several levels of intestinal barrier defence. Such abnormalities in patients and experimental models include: i) structural defects in the epithelial barrier, i.e. damaged tight junction (TJ) proteins, brush border membrane peroxidation, and an increased intestinal permeability to macromolecules [3,4]; ii) impaired innate defences, such as compromised Paneth cell synthesis of antimicrobial peptides, or impaired function of phagocytic cells, such as dendritic cells [5,6]; and iii) the overgrowth and dysbiosis of intestinal microflora due to gut hypomotility, innate defence system damage and reduced bile flow [3,7–9]. Intestinal barrier damage parallels with cirrhosis progression and is particularly severe when ascites and GBT have developed.

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Abbreviations: GBT, gut bacterial translocation; TJ, tight junction; ZO-1, zonula occludens-1; FXR, farnesoid X receptor; MLN, mesenteric lymph nodes; OCA, obeticholic acid; SHP, small heterodimer partner; OTU, operational taxonomic units; TNF, tumor necrosis factor; IL, interleukin.

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Bile acids play a pleitropic role in maintaining intestinal barrier homeostasis. The intestinal microbiota is directly affected by the bacteriostatic properties of bile acids, and indirectly via the synthesis of antimicrobial peptides and modulation of innate immunity through farnesoid X receptor (FXR) stimulation [10,11]. FXR, the nuclear receptor for bile acids, is mainly expressed in tissues frequently exposed to these molecules including the liver, intestine and kidneys. Accordingly, experimental models lacking bile acids in the intestinal lumen, such as models of cholestasis by bile duct ligation, feature intestinal bacterial overgrowth, inflammation, increased permeability and also GBT to mesenteric lymph nodes (MLN) [11–13]. This scenario is reproduced in *FXR* knockout mice and can be rescued by synthetic FXR agonists, conjugated bile acids or internal biliary drainage [11,13,14].

Cirrhosis reduces the amount of bile produced and modifies its composition. This results in decreased bile acids in the gut, as observed in patients, and in models of cirrhosis such as those induced by CCl_4 [14–16]. Interestingly, low intestinal bile acid levels have been linked to dysbiosis in patients with cirrhosis [7]. Hence, it could be that the cirrhosis-induced reduction in bile acid pool size could impair intestinal FXR axis activation, leading to intestinal dysbiosis, inflammation and barrier damage, thus contributing to GBT. Accordingly, FXR agonists, such as obeticholic acid (OCA), could restore intestinal barrier function and inhibit GBT to the MLN in rats with CCl_4-induced cirrhosis with ascites, a non-cholestatic model of cirrhosis featuring a high rate of GBT. OCA, also known as INT-747, is a first-in-class bile acid analogue, derived from chenodeoxycholic acid – the natural FXR agonist, and shows a 100-fold greater FXR agonistic activity than the natural compound [17]. The aim of this study was to investigate the long-term effects of the FXR agonist OCA on GBT, and intestinal microbiota composition, barrier integrity and inflammation in rats with CCl_4-induced cirrhosis with ascites.

Materials and methods

Experimental model of cirrhosis

Male Sprague-Dawley rats (Harlan, Horst, Netherlands) were used for all experiments. Cirrhosis was induced by weekly gavage with CCl_4 and phenobarbital in the drinking water, as described in Supplementary material. Phenobarbital-treated age- and sex-matched rats were used as the control group. All experiments were approved by and performed in accordance with the ethics committee of the University of Alcalá regulations, the Guide for the Care and Use of Laboratory Animals.

Study design

OCA, a synthetic FXR ligand, was prepared weekly by dissolving in methylcellulose, according to manufacturer's protocol (*Intercept Italia S.r.l.*, Perugia, Italy). Once ascites developed, rats were randomized to OCA (5 mg/kg·day) or vehicle (methylcellulose 0.5%) by oral gavage for two weeks [18]. Control animals were randomized into the same groups.

Instrumentation

Experiments were performed 7 days after the last CCl_4 dose. Animals underwent laparotomy under anesthesia with sevofluorane (Abbott Laboratories, Madrid, Spain) in strict aseptic conditions. Rats were shaved, the skin disinfected and the following tissues and fluids were consecutively removed: ascitic fluid using calibrated sterile syringes, blood by aortic puncture, MLN, liver and small intestine. Samples of MLN, ascites and stool of the terminal ileum were used for bacteriological study. Samples of small intestine and liver were snap-frozen and/or formalin-fixed. Mononuclear cells from blood, MLN and intestinal lamina propria were obtained.

Methods

All methods are described in the Supplementary material. In brief, GBT to MLN was assessed through conventional bacteriological culture. The FXR signaling pathway was examined by analyzing the genomic ileum expression of FXR and the *FXR* target gene, small heterodimer partner (SHP). To address intestinal epithelial integrity, we determined the genomic ileum expression of the antimicrobial peptides, angiogenin-1 and alpha-5-defensin and examined the epithelial TJ proteins, zonula occludens-1 (ZO-1) and occludin, along with fecal albumin loss. Bacterial load in ileum feces was quantified by conventional culture and qPCR, and the composition of the microbiota attached to the mucosa by latest generation sequencing. The effect of OCA on intestinal and systemic inflammation was assessed by the distribution and activation of intestinal and peripheral blood immune cells by flow cytometry, and the ileum expression of inflammation-related cytokine genes. Hepatic fibrogenesis was examined by quantifying collagen fibres by Sirius Red staining, and hydroxyproline, and the expression of pro-fibrogenic genes.

Results

Characteristics of the rats

Forty-eight out of 85 rats (56%) subjected to the CCl_4 cirrhosis induction protocol survived and developed ascites. On average, rats developed ascites 13 weeks (range, 8–22) after the initial CCl_4 dose. Once ascites developed, the 48 rats were randomized to receive the 2-week course of OCA or vehicle. Four (2 allocated to the OCA and 2 to the placebo group) of the 48 cirrhotic rats with ascites died during treatment such that the final study population was comprised of 44 animals, 21 treated with OCA and 23 with vehicle. On the day of the experiment, ascites was non-significantly reduced in the OCA-treated compared to vehicle-treated rats (Supplementary Table 1). Three rats in the OCA group and no rats in the vehicle group showed ascites reabsorption. The OCA-treated animals also showed reduced liver necro-inflammatory activity, as shown by their lower aminotransferases and bilirubin, and greater plasma albumin levels (Supplementary Table 1).

OCA reduces GBT to MLN

As shown in Fig. 1A and Table 1, OCA reduced GBT to MLN from 78.3% in vehicle-treated cirrhotic rats to 33.3% (p <0.01). Notably, OCA also reduced the frequency of *Escherichia coli* detected in the MLN of rats with GBT from 100% in vehicle-treated cirrhotic rats to 28.5% (Table 1). None of the 21 OCA-treated rats had ascites infection, which developed in 5 of the vehicle-treated animals (21.7%) and was caused by the same bacterial species identified in the MLN. None of 18 control rats treated with OCA or vehicle developed GBT.

To elucidate the mechanisms of GBT reduction by OCA, we explored its effects on the different intestinal barrier levels of defence.

OCA upregulates the FXR pathway improving epithelial integrity and intestinal permeability

We first examined the ileum FXR signaling pathway in the cirrhotic animals and the changes produced by OCA. *FXR* expression

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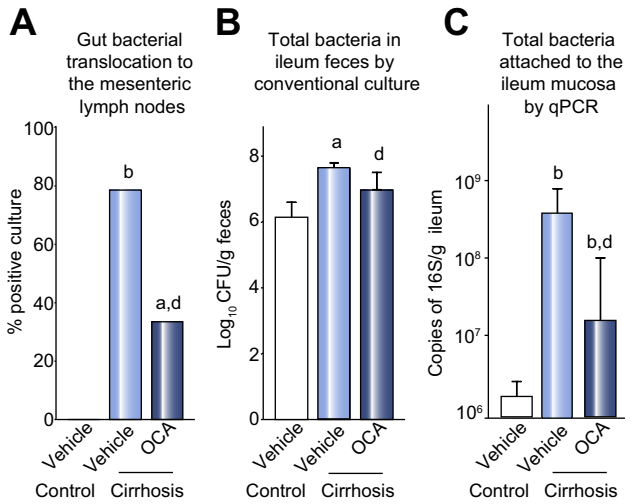

Fig. 1. Treatment with OCA reduces GBT and enteric bacterial load in cirrhotic rats. (A) GBT of aerobic bacteria to MLN quantified by conventional culture techniques. (B) Total bacteria in the fecal contents of the ileum quantified by conventional culture techniques. Data represent the mean ± SD of determinations made in 9 vehicle-treated control rats and in 23 vehicle-treated and 21 OCA-treated cirrhotic rats. CFU, colony forming units. (C) Total bacteria attached to the ileum mucosa determined by quantitative qPCR of rRNA using universal bacteria primers. Data represent the mean ± SD of determinations made in 9 vehicle-treated control and in 23 vehicle-treated and 21 OCA-treated cirrhotic rats. [a]p <0.05, [b]p <0.01 *vs.* vehicle-treated control rats; [c]p <0.05, [d]p <0.01 *vs.* vehicle-treated cirrhotic rats.

Table 1. Bacterial species isolated from MLN and ascitic fluid in vehicle- and OCA-treated cirrhotic rats.

	Mesenteric lymph nodes	Ascitic fluid
Vehicle-treated cirrhotic rats (n = 23)	*Escherichia coli* (n = 7)	-
	Escherichia coli + *Morganella morganii* (n = 2)	*Escherichia coli* (n = 1)
	Escherichia coli + *Streptococcus viridans* (n = 2)	-
	Escherichia coli + *Enterococcus faecalis* (n = 2)	*Escherichia coli* + *Enterococcus faecalis* (n = 2)
	Escherichia coli + *Proteus mirabilis* (n = 5)	*Escherichia coli* + *Proteus mirabilis* (n = 2)
	Negative (n = 5)	-
OCA-treated cirrhotic rats (n = 21)	*Escherichia coli* (n = 2)	-
	Enterococcus faecalis (n = 2)	-
	Enterobacter cloacae (n = 1)	-
	Proteus mirabilis (n = 2)	-
	Negative (n = 14)	-

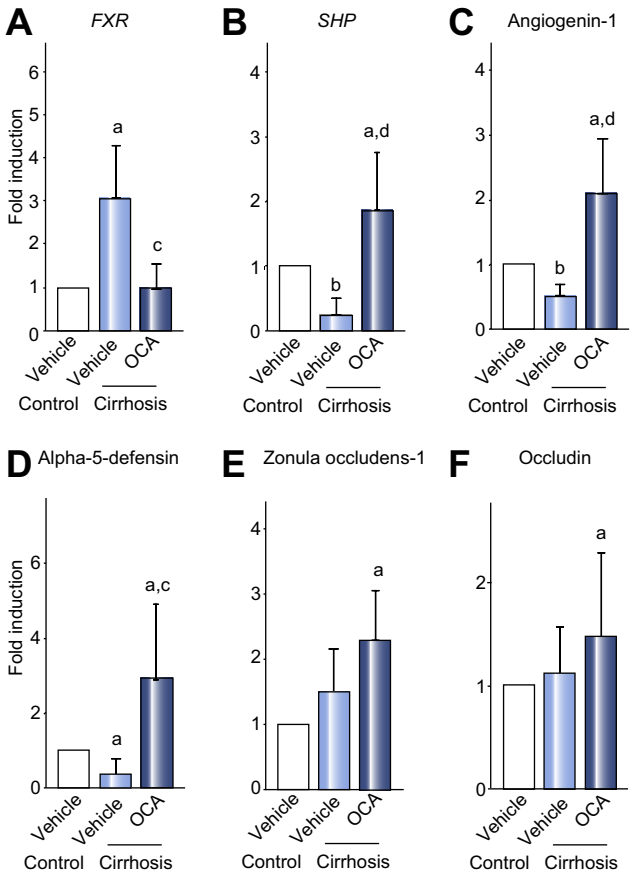

Fig. 2. Treatment with OCA increases the ileum expression of the FXR target gene *SHP*, the antimicrobial peptides angiogenin-1 and alpha-5-defensin, and the TJ proteins zonulin-1 and occludin in cirrhotic rats. Gene expression was determined by qPCR of total mRNA prepared from ileum segments. Values were normalized to *28S* mRNA. Data represent the mean ± SD of values recorded in 6–14 rats per group. [a]p <0.05, [b]p <0.01 *vs.* vehicle-treated control rats; [c]p <0.05, [d]p <0.01 *vs.* vehicle-treated cirrhotic rats.

in the ileum of vehicle-treated cirrhotic rats was 3.2-fold greater (p <0.05) compared to controls (Fig. 2A). However, the reduced activity of the FXR signaling pathway was indicated by the lower ileum expression of the *FXR* target gene, *SHP* reduced by 4-fold (p <0.01), detected in cirrhotic rats compared to controls (Fig. 2B). Interestingly, this reduction in ileum *SHP* expression was more intense in cirrhotic rats showing GBT (Table 2). Thus, OCA efficiently modulated the ileum FXR pathway in cirrhotic rats, as revealed by normalization of *FXR* expression (p <0.05) and the upregulation by 8-fold (p <0.01) of *SHP* expression (Fig. 2A–B).

We then explored the genomic expression of the intestinal antimicrobial peptide, angiogenin-1, a protein transcriptionally regulated by FXR [11]. The reduced ileum expression in cirrhotic rats of another intestinal antimicrobial peptide, alpha-5-defensin, has been reported [5]. Fig. 2C–D shows that the expression of mRNA for both peptides was lower (p <0.05) in vehicle-treated cirrhotic rats than in controls. In cirrhotic rats, OCA significantly increased this reduced ileum expression of angiogenin-1 (by 4-fold, p <0.01) and alpha-5-defensin (by 6.4-fold, p <0.05).

Intestinal epithelial integrity was assessed by ileum immunofluorescence and gene expression of epithelial TJ

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Table 2. Characteristics of vehicle-treated control rats and cirrhotic rats with and without GBT.

	Vehicle-treated control rats	Vehicle-treated cirrhotic rats	
		With GBT	Without GBT
Ileum mRNA expression (fold-induction relative to vehicle-treated control rats)	(n = 6)	(n = 12)	(n = 5)
SHP		0.13 ± 0.11[b]	0.46 ± 0.35[a,c]
Zonula occludens-1		1.1 ± 0.5	2.2 ± 0.2[a,d]
Occludin		0.7 ± 0.2	1.5 ± 0.3[a,c]
Angiogenin-1		0.36 ± 0.2[b]	0.68 ± 0.3[c]
Alpha-5-defensin		0.16 ± 0.1[b]	0.8 ± 0.4[c]
Interferon-gamma		4.13 ± 2.36[b]	1.78 ± 0.4[a,c]
TNF-α		3.14 ± 3.2[b]	1.86 ± 0.6[a,c]
IL-17A		7.42 ± 1.41[b]	3.61 ± 4.85[a,c]
IL-10		2.79 ± 2.46[a]	0.61 ± 0.01[d]
MadCAM-1		2.14 ± 1.98[a]	0.55 ± 0.77[d]
TLR-4		5.54 ± 3.04[a]	0.76 ± 0.1[d]
Ileum NF-κB activity (fold-induction relative to vehicle-treated control rats)		1.69 ± 0.6[b]	1.25 ± 0.2[a,c]
Intestinal lamina propria (cells/cm x10^{-3})	(n = 9)	(n = 18)	(n = 5)
Inflammatory monocytes (CD43^{-}NK-RP1A^{med+})	2.1 ± 0.5	7.7 ± 0.02[b]	4.4 ± 1.94[a,c]
Recently activated T-helper lymphocytes (CD134^{+})	3.2 ± 1.1	13 ± 6[b]	9 ± 10[b]
NK lymphocytes (CD3^{-}NK-RP1A^{high})	19 ± 10	35 ± 16[a]	26 ± 14
Peripheral blood (cells/ml x10^{-3})	(n = 9)	(n = 18)	(n = 5)
Inflammatory monocytes (CD43^{-}NK-RP1A^{med+})	41 ± 22	552 ± 497[b]	266 ± 260[a,c]
Recently activated T-helper lymphocytes (CD134^{+})	22 ± 30	101 ± 79[b]	82 ± 50[d]
NK lymphocytes (CD3^{-}NK-RP1A^{high})	61 ± 12	1024 ± 813[b]	420 ± 364[a,d]
Albumin in feces (ng/mg)	16.5 ± 5.9	53.6 ± 21[b]	21.1 ± 12[d]

[a]p <0.05, [b]p <0.01 *vs.* vehicle-treated control rats.
[c]p <0.05, [d]p <0.01 *vs.* vehicle-treated cirrhotic rats with GBT.

proteins. ZO-1 immunostaining of the ileum epithelium showed a continuous apical pattern in control rats, but discontinuous and redistributed from the apical border in cirrhotic rats (Supplementary Fig. 1). Although vehicle-treated cirrhotic rats showed similar genomic expression of *ZO-1* and occludin to controls (Fig. 2E–F), the expression of their coding genes was significantly lowered in cirrhotic rats with GBT than without GBT (Table 2). Notably, OCA significantly upregulated the ileum expression of *ZO-1* and occludin (Fig. 2E–F), and restored the continuous pattern and apical distribution of ZO-1 (Supplementary Fig. 1).

Finally, we assessed intestinal permeability by measuring fecal albumin loss [19]. Greater (p <0.01) feces albumin content was observed in vehicle-treated cirrhotic rats than in controls (45.5 ± 12.5 *vs.* 16.5 ± 5.9 ng/mg), being even greater (p <0.01) in those with GBT (Table 2). OCA reduced (p <0.01) the fecal albumin loss in cirrhotic rats (25.7 ± 9.9 ng/mg), indicating improved intestinal barrier function attributable to the FXR agonist treatment. Since hypoalbuminemia might affect fecal albumin, we normalized fecal albumin concentration to that of serum to overcome the possible effects of differences in serum albumin among groups, resulting in even greater differences (5.3 ± 3.1, 37.3 ± 9.3, and 11.6 ± 5.4 for vehicle-treated controls, vehicle- and OCA-treated cirrhotic rats, respectively, p <0.01).

OCA ameliorates ileum bacterial overload and dysbiosis

To gain further insight into the processes involved in GBT reduction by OCA, we investigated its impact on the ileum's bacterial load and microbiota composition. The total number of bacteria in the fecal stream of the ileum lumen of cirrhotic rats, as quantified by conventional culture techniques, was greater than in controls (7.6 ± 0.2 *vs.* 6.2 ± 0.6 logCFU/g, p <0.05). Further, this load was reduced by OCA (6.9 ± 0.5 logCFU/g, p <0.01) (Fig. 1B). We also quantified the enteric bacterial load by qPCR, focusing on the microbiota mucosal community, as the fraction showing most intimate crosstalk with the intestinal mucosa [20]. Ileum mucosa-attached total bacterial contents were 3.8-fold greater in vehicle-treated cirrhotic rats than in controls (p <0.01). These contents were also significantly (p <0.01) reduced by OCA (Fig. 1C).

We then went on to examine by latest generation sequencing the composition of the microbiota community attached to the ileum mucosa. Fig. 3A–B shows that at the phylum level, vehicle-treated cirrhotic rats displayed a reduced number of operational taxonomic units (OTU) of Firmicutes, such as *Lactobacillus*, and increased OTU of Bacteroidetes, and especially of Proteobacteria, namely *Escherichia*. However, OCA modified the distinctive enteric microbiome of cirrhotic rats. Compared with vehicle-treated cirrhotic rats, OCA-treated cirrhotic animals showed fewer OTU of Proteobacteria, especially of *E. coli*, and more OTUs of Bacteroidetes and Actinobacteria. To better understand these effects, we compared differences in the median OTU values obtained for each taxonomic group among the groups (Fig. 3B). This differential analysis revealed an abundance of *E. coli/Shigella* (phylum: Proteobacteria) to the detriment of *Lactobacillus* (phylum: Firmicutes) in the vehicle-treated cirrhotic rats, which was partly resolved by OCA.

In a principle component analysis, we also compared intestinal microbiota across groups (Fig. 3C). Vehicle- and OCA-treated cirrhotic rats clustered separately from controls. Among the cirrhotic animals, Proteobacteria, particularly *E. coli/Shigella*, emerged as the most abundant component of the ileum microbiota. The effects of OCA included expansion of the Bacteroidetes, particularly the phyla Bacteroides and Firmicutes, mostly *Lactobacillus* and *Allobaculum*.

We also examined whether a direct bacteriostatic effect of the compound could account for the differences observed on the intestinal microbiota between the OCA- and vehicle-treated cirrhotic rats. Different fecal bacterial strains were co-cultured *in vitro* in Mueller-Hinton broth containing different OCA concentrations. Results indicated no effects of OCA on bacterial growth.

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OCA rescues both intestinal and systemic inflammation

We followed our study of the mechanisms involved in GBT reduction by OCA by examining its impact on the infiltration and distribution of immune cells in the intestine. Interestingly, the intestines of cirrhotic rats were considerably inflamed, as indicated by the increased (p <0.01) numbers of inflammatory monocytes (by 3.4-fold), total (by 1.5-fold) and recently activated T-helper lymphocytes (by 4.1-fold), and T-cytotoxic lymphocytes (by 2.2-fold) identified in the lamina propria of vehicle-treated cirrhotic rats compared with controls (Supplementary Table 3). These changes at the cell level were accompanied by the increased (p <0.05) activity of nuclear transcription factor κB (NFκB) (by 1.5-fold) and the pro-inflammatory cytokines, interferon-gamma (by 3-fold), tumor necrosis factor (TNF)-alpha (by 3-fold), and interleukin (IL)-17A (by 7-fold), and anti-inflammatory cytokine IL-10 (by 2-fold) in the ileum of vehicle-treated cirrhotic rats (Fig. 4A–D). Notably, the gene expression of *MadCAM-1*, a molecule involved in intestinal leukocyte recruitment, and of the pro-inflammatory TLR-4, the LPS ligand, was also increased by 1.6-fold (p <0.05) and by 4-fold (p <0.01) in the intestines of cirrhotic rats, respectively (Fig. 4E–F). Intestinal inflammation was more severe in the vehicle-treated cirrhotic rats showing GBT (Table 2).

In cirrhotic rats, OCA treatment significantly reduced, but did not normalize, the above described cirrhosis-associated intestinal infiltration of activated immune cells (Supplementary Table 3). Notably, OCA normalized NF-κB activation and the expression of inflammation-related cytokine genes in the intestines of cirrhotic rats (Fig. 4).

Additionally, this study confirms previous evidence obtained by our group of MLN and peripheral blood inflammation in cirrhotic rats (Supplementary Table 3) [21,22]. As in the intestinal compartment, OCA significantly reduces the expansion of activated inflammatory monocytes, and effector and activated T-lymphocytes in the MLN and peripheral blood of cirrhotic rats. Serum endotoxin, which was greater in vehicle-treated cirrhotic rats than in controls (1.35 ± 0.31 *vs.* 0.49 ± 0.2 EU/ml, p <0.01), was significantly reduced by OCA (0.44 ± 0.3 EU/ml, p <0.01)

OCA associates with reduced liver fibrogenesis

We completed our study by examining whether GBT reduction by OCA was associated with changes in liver fibrogenesis. OCA decreased (p <0.01) the fibrosis area and hydroxyproline content of the liver by 1.2-fold and by 1.6-fold, respectively, in cirrhotic rats (Fig. 5A–B). The OCA antifibrogenic effect was supported by

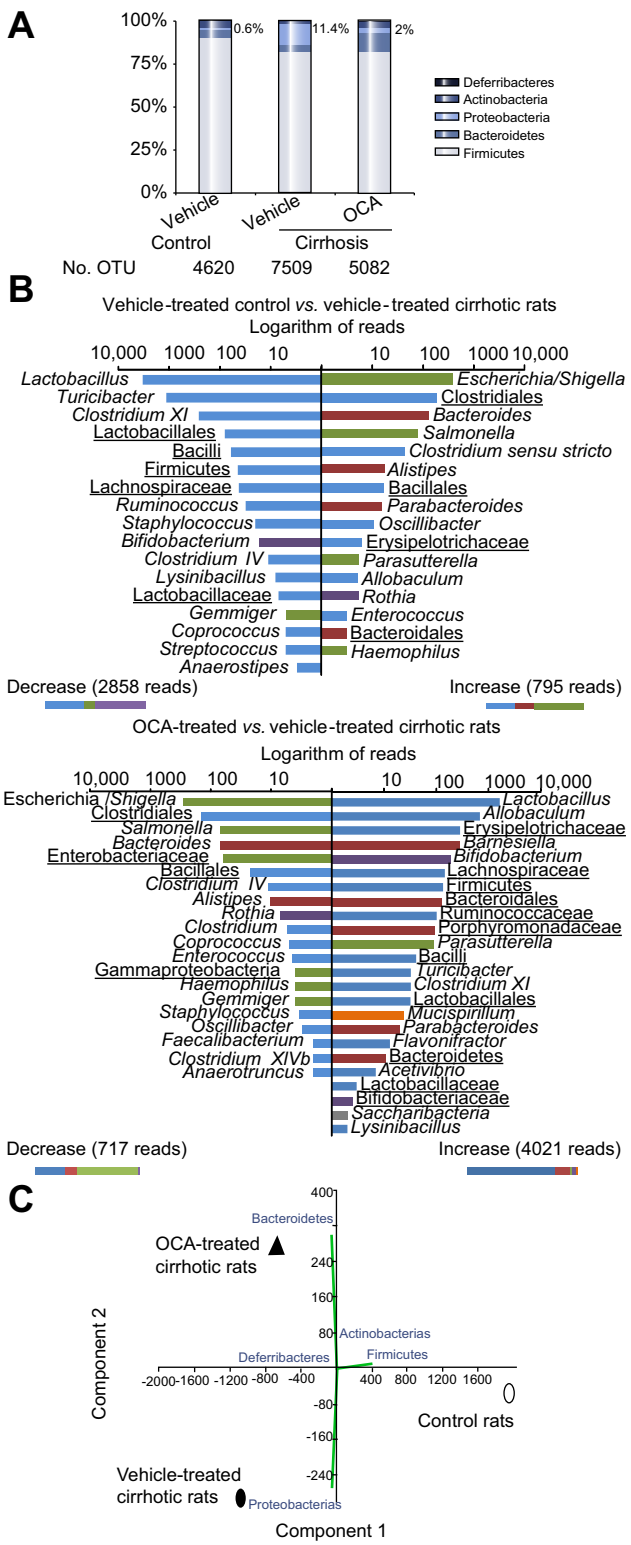

Fig. 3. Treatment with OCA resolves ileum dysbiosis in cirrhotic rats. Representative experiment-specific operational taxonomic unit (OTU) sequences (97% identity) were classified using the Ribosomal Database Project classifier and plotted. Data represent the results of experiments in 9 vehicle-treated control rats, and in 23 vehicle-treated and 21 OCA-treated cirrhotic rats expressed as medians. (A) The graph shows percentage contributions of the indicated phyla to each community. Percentages in numbers refer to Proteobacteria. (B) Microbiota (at the genera level) attached to the ileum mucosa in vehicle-treated control rats *vs.* vehicle-treated cirrhotic rats and in OCA-treated cirrhotic rats *vs.* vehicle-treated cirrhotic rats. Results are expressed as differences in medians for the vehicle-treated control rats minus vehicle-treated cirrhotic rats or OCA-treated minus vehicle-treated cirrhotic rats. The bacterial composition is related to family structures except for incomplete reads that were expressed in orders or class (underlying). Major phylum changes are also summarized at the bottom of the figure as: Firmicutes (blue), Bacteroidetes (red), Actinobacteria (purple) and Proteobacteria (green). (C) Principal component analysis conducted on weighted and normalized P1 *vs.* P2 UniFrac distances for microbiota attached to the ileum mucosa. The panel shows the clustered phyla. Each point represents the median of values recorded in 9 vehicle-treated control rats, and 12 vehicle-treated and 14 OCA-treated cirrhotic rats.

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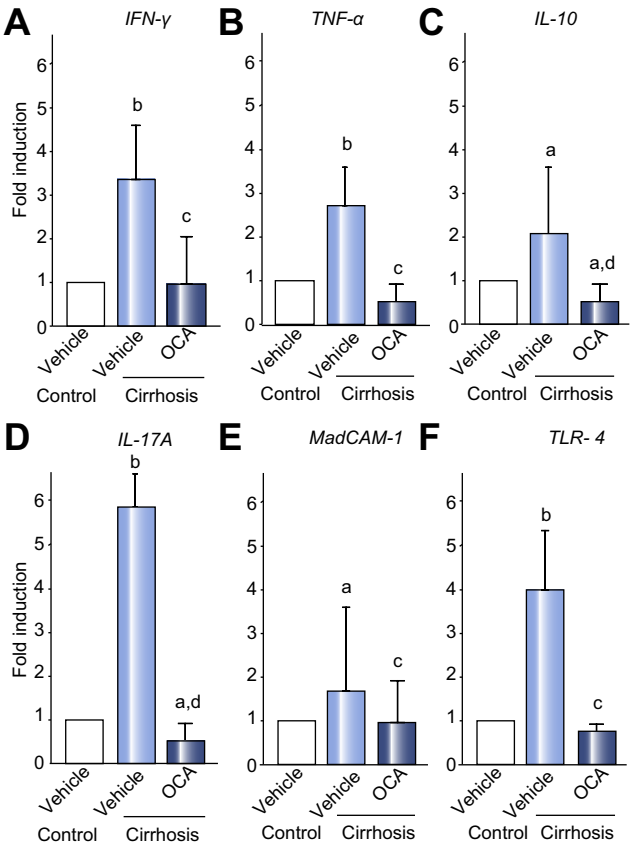

Fig. 4. Treatment with OCA reduces the ileum expression of the inflammatory cytokines interferon-gamma (INF-γ), *TNFα*, *IL-17A* and *IL-10*, *TLR-4* and the adhesion molecule *MadCAM1* in cirrhotic rats. Gene expression was determined by quantitative qPCR of total mRNA prepared from ileum segments. Values are presented after normalization to *28S* mRNA. Data represent the mean ± SD of values recorded in 6–14 rats per group. ^{a}p <0.05, ^{b}p <0.01 *vs.* vehicle-treated control rats; ^{c}p <0.05, ^{d}p <0.01 *vs.* vehicle-treated cirrhotic rats.

reductions (p <0.01) in the liver expression of the pro-fibrogenic genes procollagen-α1(I), *TIMP-1* and *TGFβ*, and the hepatic stellate cell activation marker, *α-SMA* (Fig. 5C).

OCA lacks significant effects on enteric microbiota- and epithelial inflammation-related factors in control rats

Finally, we assessed the intestinal effects of OCA in control rats, i.e. animals with normal flow of bile acids entering the intestine and without intestinal inflammation. Our observations indicate no impact of OCA on ileum FXR expression, though the expression of its reporter gene *SHP* was enhanced (Supplementary Table 4). OCA led to the increased (p <0.05) ileum expression of the antimicrobial peptides angiogenin-1 and alpha-5-defensin. The changes induced by OCA in the intestinal microbiota of control rats were of a much lower order of magnitude than those in cirrhotic rats (Supplementary Fig. 2; Supplementary Table 4).

Discussion

In this study, we show that a course of the FXR agonist OCA given to rats with CCl_4-induced cirrhosis with ascites markedly reduces the high rate of GBT distinctive of this model. OCA treatment achieves this effect through several complementary mechanisms including: i) marked reduction in fecal bacterial load; ii) partial recovery of intestinal dysbiosis; iii) improvements in both intestinal barrier function and gut inflammation; and iv) reduced liver fibrogenesis.

We tested the efficacy of OCA on GBT in rats with CCl_4-cirrhosis, a model of non-cholestatic liver disease. The capacity of OCA to reduce GBT has been previously shown in rats with cholestasis by bile duct ligation (BDL) [12]. Long-term CCl_4 intoxication induces parenchymal liver damage resembling human cirrhosis more than BDL. In our design, OCA was not initiated until ascites developed and cirrhosis had reached an advanced stage with an elevated rate of GBT. GBT pathogenesis in advanced cirrhosis involves a multiplicity of mechanisms besides intestinal lumen bile acid depletion [2]. Our experimental model and design represent a challenging preclinical setting whereby the reduction in GBT produced by OCA cannot be merely explained by the exogenous replacement of bile acids in the intestinal lumen.

FXR activation by bile acids or FXR agonists maintains the mucosal integrity by regulating the expression of a variety of genes involved in mucosa protection and defence against inflammation in rodents subjected to BDL [11,12] or dextran sulfate sodium-induced colitis [10,18]. Our results extend the reparative effect of OCA in the intestine to a model of advanced non-biliary cirrhosis with ascites. OCA restored the impaired ileum FXR pathway in cirrhotic rats, as measured by upregulation of the expression of *SHP*, the FXR reporter gene. The defective intestinal FXR activity observed in our model could be explained by the overall reduction in bile flow and decrease in bile acids, the natural FXR ligands, with worsening cirrhosis [7]. In addition, the diminished FXR pathway activity observed might be related to suppressor effects exerted by inflammatory signals or by the dysbiosis associated with cirrhosis [10,18,23], such as those found in the intestine of our model of cirrhosis. Indeed, we observed profoundly reduced FXR activity in the ileum of cirrhotic rats with GBT, animals with most severe intestinal inflammation. Interestingly, FXR pathway activation and GBT reduction were associated with the increased ileum expression of the antimicrobial peptides angiogenin-1 and alpha-5-defensin. The latter might represent a compensatory mechanism induced by FXR activation, which could be directly involved in the improvement observed in intestinal barrier function and in the bacterial translocation-reducing effect of OCA [11,18].

In response to OCA, enteric bacterial load normalized and intestinal dysbiosis resolved in cirrhotic rats. OCA corrected the intense relative abundance of Proteobacteria and the reduced relative density of Firmicutes. The distinctive dysbiosis pattern found in our model is similar to the one described in humans with cirrhosis, which has been correlated with disease severity and linked to low bile acid levels entering the intestine [7,9,24]. Our results on microbiota composition by OCA add to the reduced enteric bacterial load and GBT induced by oral conjugated bile acids in the same animal model [14]. In contrast to conjugated bile acids, the effect of OCA on intestinal microflora cannot be ascribed to a direct bacteriostatic membrane detergent effect, as shown in our *in vitro* experiment. OCA has a 100-fold greater affinity than chenodeoxycholic acid for the FXR receptor, its most active natural agonist [17]. Thus, the microbiota modulating effect of OCA appears to be related to an improvement in the intestinal barrier, including several mechanisms such as the

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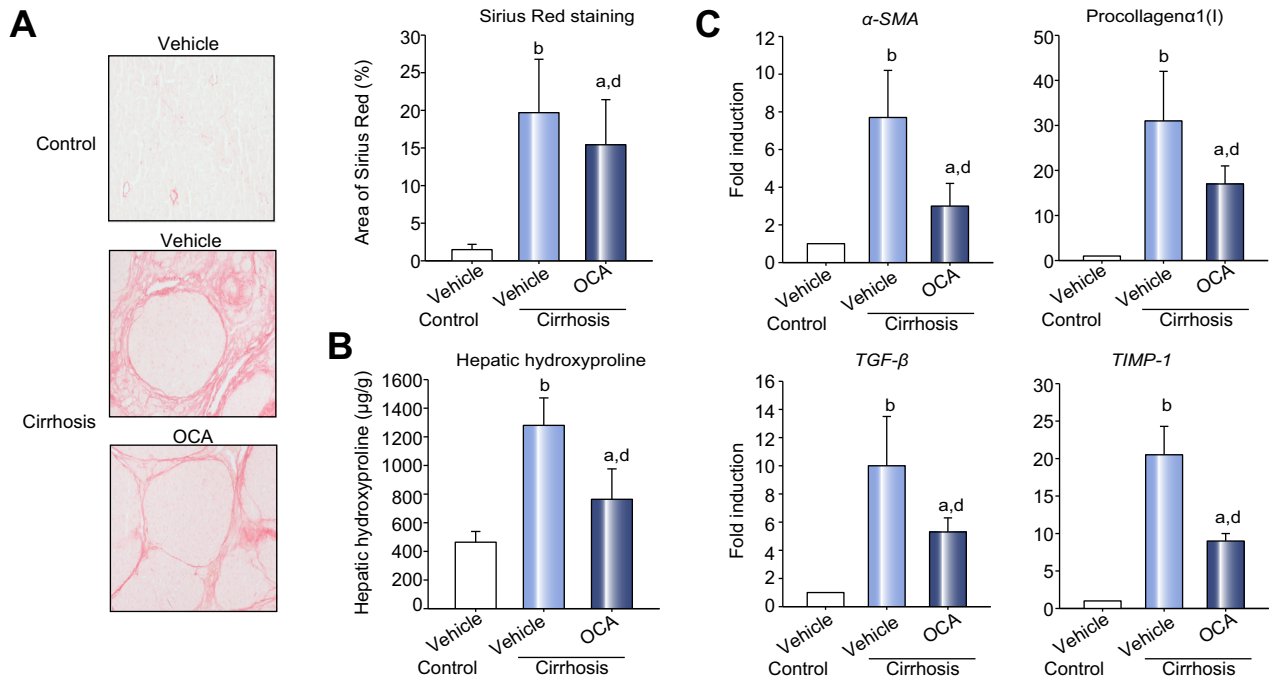

Fig. 5. Treatment with OCA reduces liver fibrosis in cirrhotic rats. (A) Collagen fibres in the liver measured as the area stained with Sirius Red (magnification 20×). (B) Hepatic collagen measured as hydroxyproline content. (C) Hepatic expression of *α-SMA*, α1-procollagen 1(I), *TGF-β*, and *TIMP-1*. Gene expression was determined by quantitative qPCR of total mRNA extracted from liver fragments. Values are expressed normalized to *28S* mRNA. Data represent the mean ± SD of values recorded in 6–14 rats per group. [a]p <0.05, [b]p <0.01 *vs.* vehicle-treated control rats; [c]p <0.05, [d]p <0.01 *vs.* vehicle-treated cirrhotic rats.

previously discussed increased expression of antimicrobial peptides and inhibition of the inflammatory response.

A remarkable finding of our study was that the well-known inflammatory systemic state in cirrhotic rats was concurrent with intestinal inflammation, as featured by an augmented number of monocytes and T-lymphocytes and the markedly increased expression of inflammation-related genes, such as interferon-gamma and IL-17A, in the ileum. The severity of intestinal inflammation was especially intense in rats with GBT, which were also those showing the lowest expression of antimicrobial and TJ proteins along with the greatest fecal albumin loss. Our findings also confirm previous observations of mononuclear cell infiltration of the lamina propria in cirrhotic rats with ascites and GBT [25,26]. The intestinal mucosa of rats with advanced cirrhosis mimics that observed in primary diseases of the intestine, such as Crohn's disease, in which mucosal immune system activation has been established as the basic mechanism of intestinal damage and increased intestinal permeability [27,28]. The increased intestinal expression of TLR-4 detected in our model and prior data from our laboratory showing that bowel decontamination with non-absorbable antibiotics suppresses intestinal infiltration by inflammatory cells in cirrhotic rats point to the intestinal flora as the stimulus leading to gut inflammation in cirrhosis [6,20,29]. In effect, the intestinal immune system in cirrhosis is continuously challenged by a microbiota that is relatively abundant in pathogenic inflammatory taxa, such as Enterobacteriaceae, and poor in benign autochthonous taxa, such as Laphnospiraceae, which mitigate intestinal inflammation [30]. In our study, OCA ameliorated intestinal inflammation and improved intestinal barrier function. This anti-inflammatory effect of OCA in cirrhotic rats might have been exerted via several mechanisms including: i) a reduction in the increased enteric bacterial load; ii) an improvement in intestinal dysbiosis involving a fall in Proteobacteria; and iii) the described direct modulatory effects on activated immune system cells [10]. In this regard, inflammatory mediators such as TNF-alpha and interferon-gamma modify the expression and function of TJ proteins leading to increased intestinal permeability, providing an explanation for the improvement in the intestinal barrier by OCA. In turn, OCA reduces the intestinal and systemic inflammation in advanced cirrhosis, further supporting the evidence for reversibility of this pathogenic mechanism, as previously shown with a biological response modifier [2,31].

The beneficial effect of OCA on intestinal barrier integrity with the resultant reduction in GBT was associated with improved hepatic fibrosis. This finding is in agreement with previous observations of reduced liver fibrogenesis by FXR ligands in models of hepatic fibrosis induced by porcine serum, BDL and CCl_4 [32,33]. The mechanisms of reduced liver fibrogenesis by OCA remain speculative, although several non-mutually exclusive explanations exist. It is likely that the effect of OCA correcting intestinal barrier damage and dysbiosis, and reducing GBT and endotoxinemia could contribute to reduced liver fibrogenesis. Bacterial products from the gut are known to activate TLR-2, -4 and -9 thus promoting inflammation, steatosis, and fibrosis [34]. Additionally, OCA has been shown to exert an antifibrotic action, which seems to be mediated by direct inhibition of hepatic stellate cell activation [33,35], and by attenuation of asymmetric dimethylarginine inhibition of nitric oxide formation [12]. Besides, the effects of OCA increasing insulin synthesis, decreasing hepatic lipogenesis and protecting hepatocytes from bile acid-induced cytotoxicity might contribute to reduced liver fibrosis [36].

In conclusion, our results indicate that OCA reduces GBT by targeting several complementary mechanisms that compromise

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the function of the intestinal barrier in advanced cirrhosis. The beneficial effects of OCA at the intestinal level include increased innate defences, reduced bacterial load, recovery of dysbiosis, and inhibition of gut inflammation. In addition, amelioration of intestinal inflammation by OCA was associated with reduced systemic inflammation and liver fibrogenesis. These preclinical data provide direction for future studies designed to address the use of OCA in clinical settings of cirrhosis in which survival is determined by a high-risk of bacterial infection and inflammation of gut origin.

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Conflict of interest

All authors have nothing to disclosure.

Authors' contributions

MU, conducted the experiments, analyzed data and drafted the manuscript. ML, LM, and MJB did the experiments. MR and LG did PCR and immunohistochemistry analysis. ASD and RC analyzed the microbiota data. LL, OP and DD provided technical assistance. AA and MA obtained funding, designed the project and wrote the manuscript.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2015.12.010.

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Author names in bold designate shared co-first authorship

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