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INTESTINAL INFLAMMATION ALTERS THE EXPRESSION OF HEPATIC BILE ACID RECEPTORS CAUSING LIVER IMPAIRMENT

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Abstract

Objectives: The gut-liver axis has been recently investigated in depth in relation to intestinal and hepatic diseases. Key actors are bile acid (BA) receptors, as farnesoid-X-receptor (FXR), pregnane-X-receptor (PXR) and G-protein-coupled-receptor (TGR5), that control a broad range of metabolic processes as well as inflammation and fibrosis.

The present study aims to investigate the impact of intestinal inflammation on liver health with a focus on FXR, PXR and TGR5 expression. The strategy to improve liver health by reducing gut inflammation is also considered. Modulation of BA receptors in the inflamed colonic tissues of Inflammatory Bowel Disease (IBD) pediatric patients is analyzed.

Methods: A dextran-sulphate (DSS) colitis animal model was built. Co-cultures with Caco2 and HepG2 cell lines were set up. Modulation of BA receptors in biopsies of IBD pediatric patients was assessed by Real-time-PCR and immunohistochemistry.

Results: Histology showed inflammatory cell infiltration in the liver of DSS mice, where FXR and PXR were significantly decreased and oxidative stress was increased. Exposure of Caco2 to inflammatory stimuli resulted in the reduction of BA receptor expression in HepG2. Caco2 treatment with dipotassium glycyrrhizate (DPG) reduced these effects on liver cells. Inflamed colon of patients showed altered FXR, PXR and TGR5 expression.

Conclusions: this study strongly suggests that gut inflammation affects hepatic cells by altering BA receptor levels as well as increasing the production of pro-inflammatory cytokines and oxidative stress. Hence, reducing gut inflammation is needed non only to improve the intestinal disease but also to protect the liver.

Key words: gut, liver, bile acid receptors, inflammation, inflammatory bowel disease

What is known:

- Gut-liver axis (GLA) connects liver and intestine via bile acid (BA) metabolism.
- BA receptors, regulating lipid and glucose metabolism, are currently known to be also involved in immune and inflammatory response.

What is new:

- Gut inflammation alters farnesoid-X-receptor (FXR), pregnane-X-receptor (PXR) and G-protein-coupled-receptor (TGR5) levels and increases the production of pro-inflammatory cytokines and oxidative stress in the liver.
- The amelioration of intestinal inflammation causes liver improvement as shown by the BA receptor level restitution.
- Children with Inflammatory Bowel Disease show altered FXR, PXR and TGR5 expression in the inflamed colon.

Introduction

The gut and the liver are anatomically connected by portal circulation, and their functional unit realizes the gut-liver axis (GLA) with the integrity of intestinal barrier crucial for the maintenance of liver homeostasis (1-3). In this mutual relationship, the liver acts as a second firewall towards potentially harmful substances translocated from the gut, and in turn is implicated in the regulation
of the mucosal barrier (4).

The GLA connects the liver with the intestine via bile acid metabolism (5-6). Bile acids (BAs) are amphipathic steroid acids, synthesized from cholesterol in the liver, that regulate lipid, glucose and energy metabolism (7-9). Moreover, a specific role of BAs as immunomodulators is emerging (10-15).

The regulatory functions of BAs are predominantly mediated by the bile receptors, such as the nuclear receptors farnesoid-X-receptor (FXR) and pregnane-X-receptor (PXR) as well as the membrane G-protein-coupled-receptor (TGR5) (16,17).

Nuclear receptors are ligand-dependent transcription factors that regulate a variety of physiological processes, such as homeostasis, reproduction, development, inflammation and metabolism, by inducing the transcription of target genes. (16). FXR is highly expressed in the liver and gut and affects lipid and glucose metabolism (18). Similarly, PXR is involved in the regulation of xenobiotic metabolism (19), although, recent evidences outline its role also in inflammatory response, cell proliferation and migration (20).

TGR5 is a member of G-protein-coupled-receptors (GPCRs), ubiquitously expressed in diverse tissues, including endocrine organs, muscle, adipose tissue, immune cells, and intestinal tract (21). Recent literature has extended its functions to more than metabolic regulation, which include inflammatory response, cancer and liver regeneration. (21-23). Upon binding with lithocholic acids (LCA) and taurolithocholic acid (TLCA) it is activated to transduce signal transduction into the nucleus and control diverse gene expression (24).

The enterohepatic circulation of BAs is governed by specific transporters expressed in the liver and the intestine and plays a critical role in the digestion of fats and oils. During this process, the majority of the BAs secreted from the liver is reabsorbed in intestinal epithelial cells via the apical sodium-dependent bile acid transporter (ASBT/SLC10A2) and then transported into the portal vein (25).

In this setting, BAs and their receptors are attractive candidates for therapeutic development in chronic diseases such as type 2 diabetes, hypertriglyceridemia, obesity, non-alcoholic steatohepatitis, inflammatory bowel disease (IBD) and sclerosing cholangitis (26-30).

To deeper investigate the strict relationship between the liver and the gut, the present study aims to assess in vivo and in vitro the impact of gut inflammation on liver health by focusing on the modulation of BA receptors FXR, PXR and TGR5. The strategy to improve liver health by reducing gut inflammation is also considered. Moreover, given the role of BA receptors in controlling intestinal mucosal immunity and inflammation, a secondary goal is to analyze the expression pattern of FXR, PXR and TGR5 BA receptors as well as the ASBT transporter in the inflamed colonic tissues of pediatric patients with IBD and in controls.

Methods:

Ethic Statement: This work has been approved by the Ethic Committee of the Umberto I Hospital, Sapienza University of Rome, Italy. All parents of patients entered into the study provided written informed consent. Experimental procedures on mice were previously approved by the Ministry of Health.

Cell lines: The human colon adenocarcinoma cell line Caco2, the hepatic adenocarcinoma cell line HepG2 and the murine macrophage-like cell line RAW264.7 were purchased from ATCC (Rockville, MD, USA). Inflammation was induced by cytomix, a combination of TNF-alpha (10ng/ml; Sigma, St. Louis, MO, USA) and Interferon-gamma (250ng/ml).

Co-culture system: Cells will be seeded for differentiation on ThinCert cell culture polyethylene terephthalate (PET) capillary pore membranes (0.4 um pore diameter; Greiner Bio-One International GmbH) and maintained in complete medium supplemented with 10% FBS in both apical (AP) and basolateral (BL) compartments. For co-culture experiments, Caco-2 cells differentiated on filter inserts will be transferred to culture plates containing confluent HepG2 cells. Treatment with cytomix will be added to the AP compartment of Caco-2 cells and the culture plates will be incubated at 37 °C for 24 and 48 hours.

Alternatively, Caco2 cells were infected with LF82 strain at a MOI of 10:1 for 3 hours. Then medium with bacteria were removed and replaced with new medium for additional 3 hours.

Trans-epithelial Electric Resistance (TEER) assay: CACO2 cells were grown on polyethylene terephthalate membrane inserts, pore size 0.4 mm (Falcon, Becton Dickinson, Franklin Lakes, NJ). TEER values were measured using a Millicell-ERS voltohmmeter (Millipore, Billerica, MA) according to the Manufacturer's instructions.

Animal: C57BL/6 female mice (8 to 9 weeks of age) were purchased from the animal housing unit of Harlan Laboratories, SRL. Induction of colitis was performed through administration of dextran sodium sulphate (DSS, molecular mass, 36,000–50,000 Da, MP Biomedicals, Santa Ana, CA), 3% dissolved in autoclaved drinking water, for 7 days. Clinical score (CS) was assessed according to the criteria of Maxwell et al (31). The 7th day, animals were euthanized. Distal colonic, and liver specimens were fixed immediately in a 10% (w/v) formalin solution for histological analysis and frozen in liquid nitrogen or for molecular analyses.

Patients: 10 patients with Crohn's Disease (CD) (median age: 13.0 years; range: 6-17 years), 10 with Ulcerative Colitis (UC) (median age: 12.9 years; range: 7-17 years), and 10 controls (median age: 11 years; range: 5-17 years), referred to the Maternal Infantile Department and Urological Sciences, at the Sapienza University of Rome and needed an ileo-colonoscopy to reassess the intestinal disease, were included in this study. All selected patients had an established diagnosis of IBD and were in active phase of disease even under therapy. Patients were under treatment with immunomodulators (azathioprine or methotrexate), mesalamine, or oral corticosteroids at low doses. Activity in CD and UC was measured respectively by the PCDAI (Pediatric Crohn's Disease Activity Index) (32) and the PUCAI (Pediatric Ulcerative Colitis Activity Index) score (33). The intestinal inflammation was assessed at endoscopy by using the SES-CD score (34) and the endoscopic Mayo subscore (35) in CD and UC patients,

respectively. Children with incapacitating functional gastrointestinal disorders requiring extensive investigation, having normal endoscopy and histology, served as controls.

Biopsy treatment: Colonic mucosal specimens were immediately snap frozen in liquid nitrogen for RNA analysis or fixed immediately in a 10% (w/v) formalin solution for histology and immunohistochemistry.

Real-time PCR: Total RNA was isolated from cells and biopsies using the RNeasy kit (QiaGen, Hilden, Germany). Total RNA (1 ug) was reverse-transcribed to cDNA by a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR amplification was done with an ABI PRISM 7300 Sequence Detection System using the SYBR Green kit (Applied Biosystems). The quantity of each mRNA to a reference gene was calculated by the $2^{-\Delta\Delta CT}$ method.

Immunohistochemistry: Sections (4 μm) of paraffin-embedded intestinal inflamed tissues from were prepared following standard protocol. Anti-FXR and anti-TGR5 (Abcam, Cambridge, UK) antibody were used. Finally, sections were stained with hematoxylin and eosin.

Statistics: A minimum of 5 mice per group were included in all experiments. Statistical analysis for significance was determined using the GraphPad InStat software. The Kolmogorov–Smirnov test showed significant departures from the normal distribution for some of the analyzed parameters. Therefore, comparison of the group was performed by the Mann- Whitney U test. For in vitro experiments the Student's t-test was used. Experiments were repeated 3 times. Data were presented as mean ±SD. Differences were noted as significant *$P < 0.05$, and **$P < 0.01$.

Results

FXR, PXR and TGR5 levels are significantly altered in the inflamed colon as well as in the liver of mice with DSS-induced colitis

We used the 3% DSS-treated mice as a model of severe colitis. The occurrence of gut inflammation was confirmed by histology which showed the loss of crypt structure, extensive inflammatory cell infiltration and thickening of the mucosa with abundant edema (Fig.1A). Interestingly, increased inflammatory cells were also detected in the hepatic tissue of DSS mice as compared to controls (Fig.1B). Moreover, as expected, inflamed colonic tissues showed increased levels of pro-inflammatory cytokines, TNF-α, IL-6, and IL-1β ($p<0,01$), however, IL-6, and IL-1 β were strongly increased also in the liver ($p<0,01$) (Fig.1C).

FXR and PXR mRNA expression was significantly decreased in the inflamed colon of DSS-treated mice ($p<0.05$), while an increased expression of TGR5 was found ($p<0.05$) (Fig. 1 D). This finding could be explained by recent data reporting the occurrence of higher concentration of TGR5 in macrophages, that are highly involved in the tissue inflammation processes (36). To deeper investigate this point, we performed immunohistochemistry on mucosal colonic samples of mice and found that while FXR was mainly localized in the nuclei of enterocytes, TGR5 was largely confined to phagocytes (Fig. 1 E). Then, we used the murine macrophages RAW 264.7 to confirm in vitro that inflammation enhances the levels of TGR5 receptor. RAW 264.7 cells were exposed to a mix of pro-inflammatory cytokines (cytomix: TNF α +INFγ) for 6 or 24 hours. Results showed a significant increase of TGR5 ($p<0,01$), while FXR and PXR were very poorly expressed (data not shown). Accordingly, intestinal epithelial cells Caco2 and liver cells HepG2 exposed to the same inflammatory agents showed a decrease of all BA receptors, including TGR5 ($p<0,05$) (Fig. 1 F).

Remarkably, we observed a significant decrease of FXR and PXR in the liver of mice ($p<0.05$) and a substantially unchanged level moderate decrease of TGR5 as well (Fig.1 G).

Since it is increasingly recognized that FXR has antioxidant activities, in order to better characterize the liver impairment induced by intestinal inflammation, we also analyzed in hepatic tissues the gene expression of nitric oxide synthase (iNOS) and cyclooxygenase (COX2), that have been shown to play pivotal roles in the development of inflammatory diseases, including cancer. We found that both enzymes were importantly up-regulated ($p<0.01$) in the liver of DSS-treated mice (Fig.1 H).

Co-cultures of intestinal and hepatic cells show that gut inflammation directly affects liver cells by altering BA receptor expression

We set up a co-culture system with differentiated and oriented Caco2 (above) and HepG2 (below) (Fig.2 A). The full confluence of Caco2 to form a whole intestinal barrier was proven by the trans epithelial electric resistance (TEER) assay (Fig.2 B). Intriguingly, we observed upon exposure of only Caco2 to the cytomix (24, 48 hours) inflammation in HepG2 cells occurred, as shown by the increase of IL-8 and IL1- β ($p<0.01$), and in the reduction of BA receptor expression ($p<0,05$) (Fig.2 C).

Since the persisting intestinal inflammation is usually associated to bacterial dysbiosis that is characterized by a substantial increase of bacterial groups with higher pro-inflammatory potential, such as the adherent-invasive Escherichia coli (AIEC) strains, we repeated the above experiment using the AIEC prototype, LF82, as inflammatory agent. Thus, Caco2 were challenged with LF82 (MOI10:1) for 3 hours. Again, this exposure resulted in the induction of inflammation in HepG2 cells, as shown by the increase of cytokines ($p<0.01$), and in the reduction of BA receptors ($p<0,05$) that returned to normal levels after LF82 removal (FIG.2D).

Reducing gut inflammation through the anti-inflammatory agent dipotassium glycyrrhizate (DPG) protects liver health by improving FXR and PXR expression

DPG has been already studied and used in vitro and in vivo in our laboratory for its potent anti-inflammatory effects on cells from different tissues, including the gut. In this study, only Caco2 were exposed to cytomix for 48 hours or co-exposed to cytomix and DPG (150 and 300uM). We observed that DPG strongly reduced inflammation in HepG2, as shown by the decrease of IL-8 ($p<0.01$), and significantly increased FXR and PXR expression dose proportionally ($p<0.05$) (Fig 3).

The mRNA expression of FXR, PXR and TGR5 as well as transporter ASBT is notably altered in the inflamed colon of children with IBD

There is a growing body of interest about a supposed role of BAs in mucosal immunity and inflammation. To assess a possible contribution of BA receptors in chronic inflammation, we analyzed the expression pattern of FXR, PXR and TGR5 in inflamed colonic areas from children with CD, UC and in age-matched controls. Moreover, we analyzed the expression of ASBT transporter that represents the first step in bile acid reabsorption to the liver from the intestine. We found a strong decrease of FXR, PXR ($p<0.05$) and ABST ($p<0.01$) in the inflamed mucosa of all patients as compared to controls. We also found an increased expression of TGR5 ($p<0.05$) in the same patient samples as compared to controls (Fig. 4A, B), in agreement with results previously obtained in murine inflamed colonic tissues (Fig.1 D). Accordingly, immunohistochemistry showed that FXR was mainly concentrated in enterocytes and TGR5 in phagocytes (Fig. 4 C).

Discussion

Behind their role in nutrients absorption, BAs act as signaling molecules, activating several receptors that regulate central metabolic pathways but also may modulate inflammation.

Accumulating data identify BAs and their receptors as pleiotropic signaling molecules that control gut-liver crosstalk (37).

In this study, we first focused on the relationship between the gut and the liver, a close functional and vascular association also known as GLA, examining whether the occurrence of intestinal inflammation might affect healthy hepatic cells. Hence, we analyzed the liver of mice with a DSS-induced colitis and found that intestinal inflammation alters the BA receptor expression both in the inflamed colon and in the apparently normal hepatic tissue. Indeed, the cytokine analysis proven the presence of a certain grade of inflammation also in the liver, as confirmed by histology. Interestingly, the nuclear receptors FXR and PXR were markedly down-regulated both in the gut and liver samples, while the trans-membrane receptor TGR5 significantly increased in the gut but not in the liver of mice.

Recent data reported that TGR5 is highly expressed in monocytes/macrophages (36,38,39), members of the mononuclear phagocyte system that circulate through the blood and extravasate into inflamed tissues. We performed immunohistochemistry and confirmed that TGR5 is principally localized in the macrophage infiltrate of murine colonic samples, while FXR is mainly found in the nuclei of enterocytes (in uninflamed tissue). To better investigate this different cell response, we induced in vitro inflammation in intestinal epithelial cells, hepatic cells and macrophages and found that TGR5 was significantly increased only in the latter. This confirms that TGR5 is primarily expressed by macrophages and explains its marked increase in the inflamed gut, harboring the largest pool of macrophages.

Since it is recognized that FXR has also antioxidant activities (40,41), we believe that the reduction of FXR in liver cells of DSS mice may also contribute to raise the oxidative stress level, as shown by the increased expression of COX2 and iNOS.

In summary, we found that gut inflammation induced by DSS alters BA receptor expression and causes liver inflammation in mice. However, it is conceivable to attribute a share of

damage to a direct action of the DSS that reaches the liver through the portal system. Therefore, in order to assess the extent of liver damage caused by intestinal inflammation as such, we used co-cultures of intestinal and hepatic cells. Intestinal cells, grown as a confluent monolayer mimicking the gut barrier, were exposed to a mix of cytokines (TNF- α, INF-γ) as inflammatory agents. We found that the hepatic cells grown below showed increased levels of pro-inflammatory cytokines (IL-1 β, IL-8) and a down-regulation of BA receptor expression, confirming that gut inflammation is able to elicit liver impairment.

Furthermore, there is a current widely agreed view that the gut microbiota (GM) plays a central role in BA host metabolism by regulating their deconjugation, dehydroxylation, dehydrogenation and that BAs and GM reciprocally control each other's compositions (42,43). Additionally, it is known that intestinal inflammation is often associated with GM alterations leading to intestinal dysbiosis with a prevalence of adherent-invasive E coli (AIEC) pathotypes with pro-inflammatory properties (44). Hence, we used the AIEC prototype LF82 as an alternative agent to induce inflammation in intestinal cells and observed the same effects on liver cells as above. These results confirm that the onset and development of gut inflammation, featured by circulation of pro-inflammatory cytokines and abundance of AIEC strains, is also turned into a liver damage that should be seriously taken into consideration given the chronic nature of some inflammatory processes, such those characterizing the IBD.

Providing further evidence, we exposed intestinal cells to DPG, a salt of the glycoconjugated triterpene glycyrrhizin that exhibits potent anti-inflammatory, antiviral and antiallergic effects (45, 46) and was previously used by our group to counteract gut inflammation (47). Remarkably, we observed that decreasing intestinal inflammation caused the reduction of liver inflammation and the normalization of BA receptor expression, strenghtening the close gut-liver relationship.

BAs interact directly with a variety of transmembrane and nuclear receptors, in particular, FXR and TGR5 contribute to maintain the tolerogenic state of the liver and intestine immunity, indeed, they are highly expressed in innate immunity cells including intestinal and liver macrophages. Accordingly, perturbed BA circulation and/or metabolism seem to be implicated in the pathogenesis of primary sclerosing cholangitis, metabolic syndrome, colon cancer and IBD (14, 15). Thus, we analyzed the expression pattern of FXR, PXR and TGR5 in inflamed colonic tissues of CD and UC children and found that FXR and PXR were significantly decreased, while TGR5 increased, in patients as compared to age-matched controls, in agreement with results in mice. Furthermore, since BAs secreted from the liver are mostly reabsorbed in intestinal epithelial cells via ASBT, we analyzed ASBT expression in the same samples and found that it was strongly decreased. We speculate that FXR, PXR and ASBT decrease may result in intestinal BA accumulation worsening gut inflammation and causing liver distressing.

In conclusion, this study clearly demonstrates that gut inflammation, featured by the decrease of intestinal FXR, PXR and ASBT expression and increase of TGR5, is able to affect hepatic cells by altering BA receptor levels and increasing the production of pro-inflammatory cytokines and oxidative stress. Hence, reducing gut inflammation is mandatory non only to improve the intestinal disease but also to protect the liver.

Future work will be addressed to understand whether gut inflammation is involved in the progression of liver diseases.

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FIGURE 1. (A, B) Histological sections of colon and liver in DSS mice and control. (C) mRNA expression of IL-6, TNF- α and IL-1 β in colon and liver and (D) mRNA level of FXR, PXR and TGR5 in colon of DSS mice and control. (E) Immunohistochemistry showing FXR and TGR5 in colon of DSS mice and control. (F) mRNA expression of FXR, PXR and TGR5 in Caco2 and HepG2 and TGR5 level in RAW 264.7. (G,H) mRNA analysis for FXR, PXR and TGR5 and for iNOS and COX2 in liver of DSS mice and control. *P<0,05; **P<0,01.

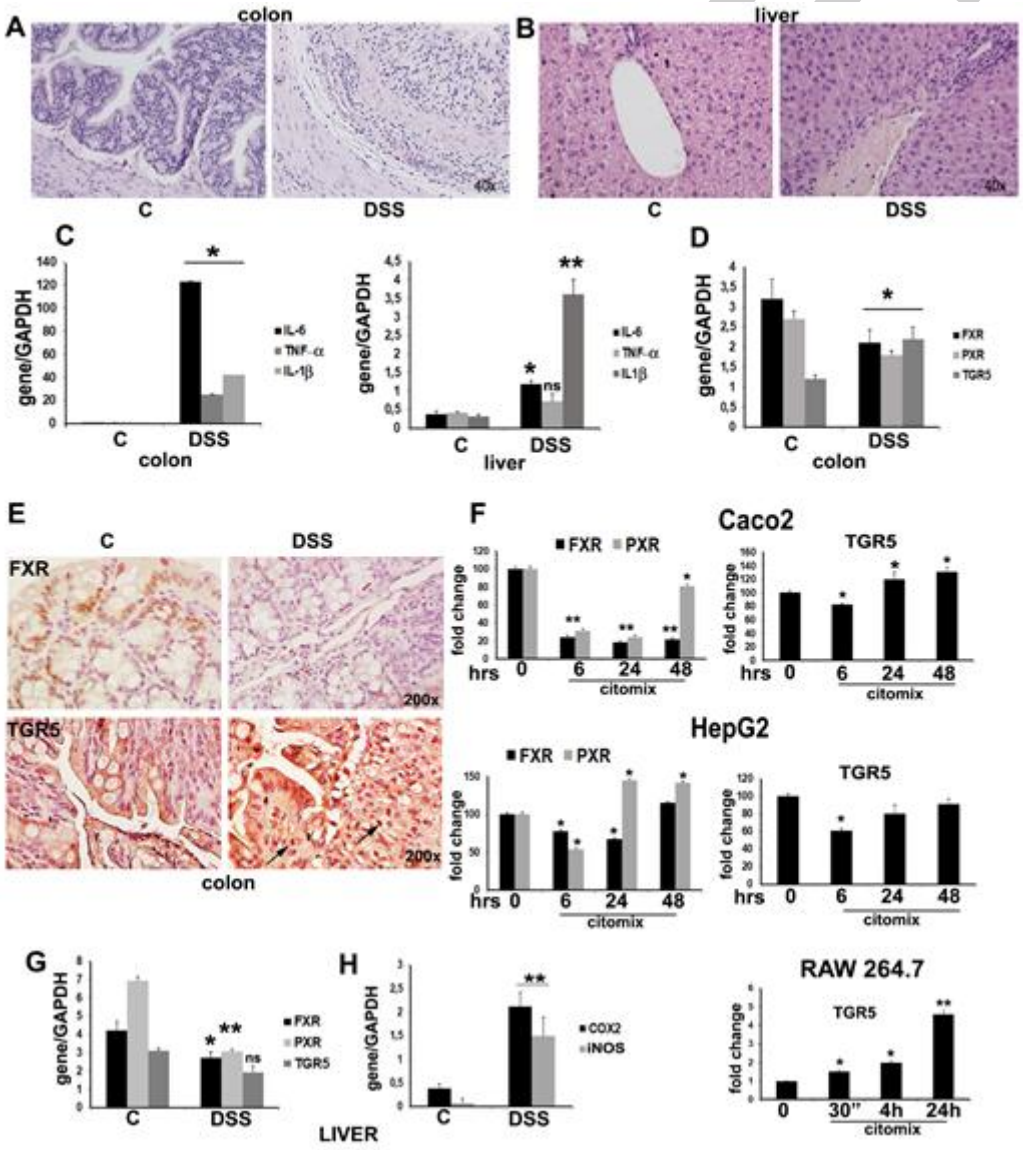

FIGURE 2. (A) Schematic representation of the co-culture model. (B) TEER measurement to control the integrity of epithelial barrier. (C) mRNA expression of IL-8 and IL-1 β cytokines and FXR, PXR and TGR5 receptors in HepG2 after 24 and 48 hours of treatment of Caco2 with cytomix. (D) mRNA expression of IL-8, IL-1 β and FXR, PXR, TGR5 in HepG2 after 3 hours of LF82 infection and after 3 hours post-infection. *P<0,05; **P<0,01.

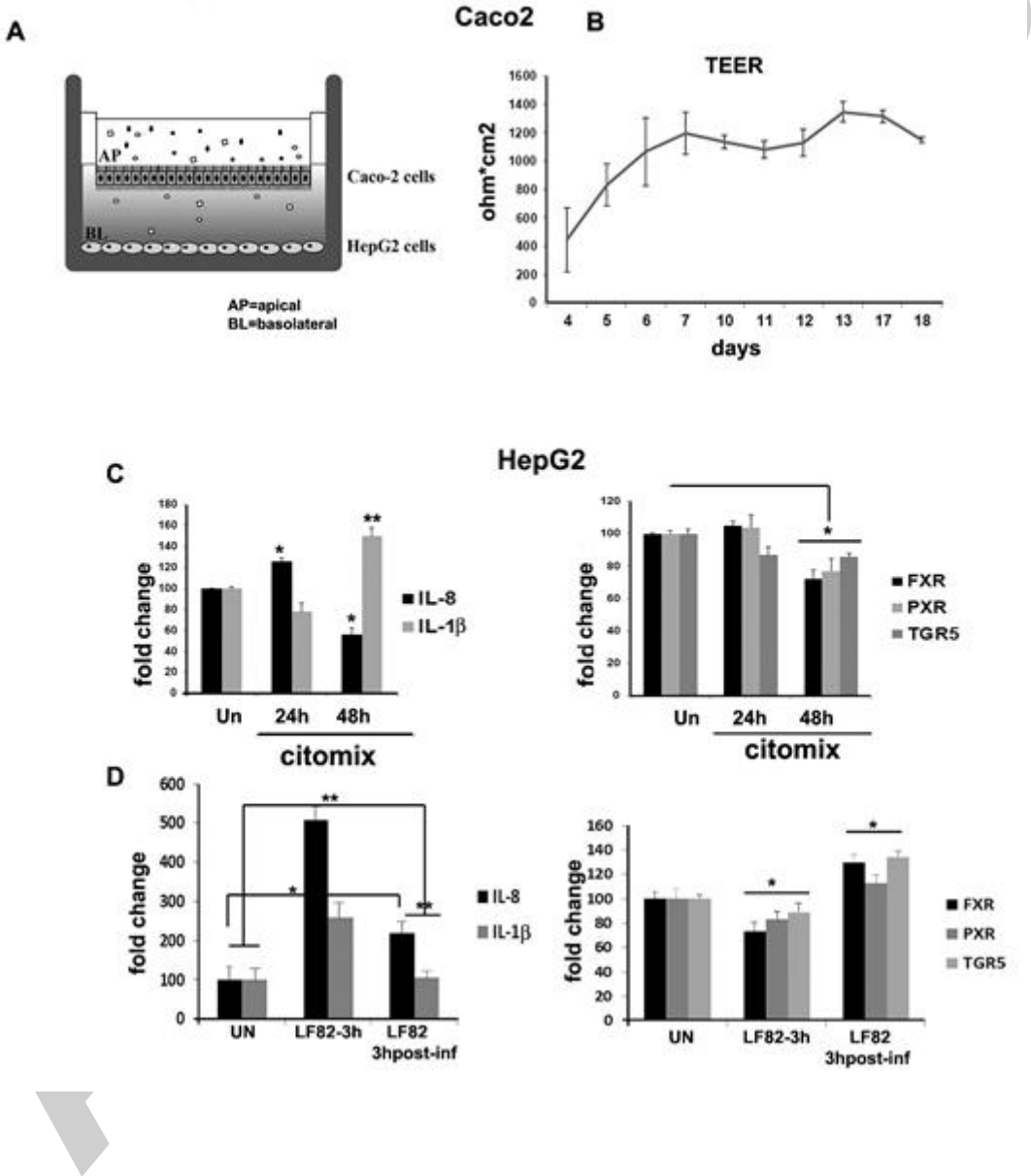

FIGURE 3. FXR, PXR and IL-8 expression in HepG2 cells after a combined treatment of Caco2 with cytomix and two concentrations of dipotassium glycyrrhizate (DPG) (150 and 300uM) in a co-colture system. *P<0,05; **P<0,01.

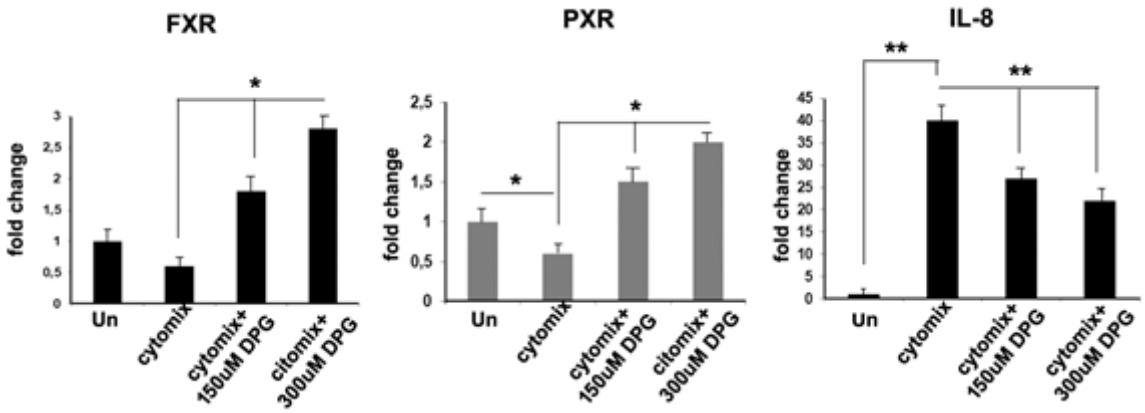

FIGURE 4. (A, B) Real-time PCR for the expression of FXR, PXR, TGR5 and IL-8 in inflamed colon of Crohn's Disease and Ulcerative Colitis pediatric patients. (C) mRNA level of ASBT in inflamed colon of Crohn's Disease and Ulcerative Colitis pediatric patients. (D) Immunohistochemistry for FXR and TGR5 in inflamed colon of a UC patients.
CD= Crohn's Disease; UC= Ulcerative Colitis. *P<0,05; **P<0,01.