Intrahepatic interleukin 10 expression modulates fibrinogenesis during chronic HCV infection

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Abstract

Introduction
Liver fibrosis is a result of continuous damage to the liver combined with accumulation of the extracellular matrix and is characteristic of most chronic liver diseases such as hepatitis C virus (HCV) infection.

Methods
This study evaluated interleukin 10 (IL10) expression in the liver and plasma of 45 HCV patients and its association with the pathogenesis and progression of liver fibrosis. The expression of transforming growth factor beta (TGFβ1) was also assessed. Patients were divided into three groups according to the METAVIR classification (F0-F1, F2 and F3-F4); there was also a control group (n = 8).

Results
In the control group, high intrahepatic IL10 mRNA expression showed a positive association with F0-F1 fibrosis, no inflammation, low concentrations of liver enzymes and a high viral load; conversely, low intrahepatic IL10 mRNA expression showed a negative association with fibrosis progression. Intrahepatic TGFβ1 mRNA expression was greater in the HCV group than in the control group, and regarding different disease phases, its expression increased as fibrosis evolved to more severe forms.

Conclusion
Intrahepatic IL10 mRNA expression decreases with persistent fibrosis, probably due to the production of TGF-β1, a potent antimitotic and fibrogenic cytokine. IL10 restricts and decreases the immune response and limits the fibrogenic response; however, a decrease in IL10 favors persistent inflammatory infiltrate, resulting in severe fibrosis.
1. Introduction

Liver fibrosis is a result of chronic damage to the liver combined with extracellular matrix accumulation and is characteristic of most types of chronic liver disease. Regardless of the initial cause, continued liver injury causes inflammatory damage, matrix deposition, parenchymal cell death and angiogenesis, leading to progressive fibrosis. The healing matrix accumulates very slowly; however, once cirrhosis is established, the potential to reverse this process is decreased, and complications develop [1]. The liver is a highly immunotolerant organ with high physiological relevance, therefore, the deregulation of the effector and suppressor immune response can induce the persistence of HCV [2]. The cytokines IL-10 and TGF-β1 contribute to an immunotolerant state of the liver, as they inhibit the differentiation of dendritic cells and promote the conversion of naïve CD4 + T cells into regulatory T cells [2, 3]. TGF-β1 is the main cytokine mediator of tissue repair, inducing myofibroblast differentiation, formation of the extracellular matrix, proliferation of fibroblasts and collagen synthesis [4]. IL-10 is an important immunoregulatory and anti-inflammatory cytokine that is produced after antigenic stimulation by several cell populations, including Th2 and Th0 lymphocytes, B cells, dendritic cells and monocytes [5]. IL-10 activity is mediated by its specific cell surface receptor IL10R, which is expressed on a variety of cells, especially immune cells [6]. Studies have shown that IL-10 inhibits the proliferation of both Th1 and Th2 cells via cellular anergy [7]. Due to its immunosuppressive activity, IL-10 reduces the effective immune response against various pathogens, favoring its persistence in the body [8]. In the liver, IL-10 production has been documented in hepatocytes, sinusoidal endothelial cells, Kupffer cells, hepatic stellate cells and resident lymphocytes, with prevalent production in the normal liver [9]. IL-10 is related to changes in the inflammatory immune response in viral hepatitis, autoimmune hepatitis, alcoholic liver disease and its effects have been demonstrated through experimental models [10]. There is evidence that patients with a strong Th1 response during acute HCV infection can eliminate the virus, whereas patients with a Th2 response (elevated IL-10 level) may progress to chronicity [11]. In addition, during treatment after liver transplantation, IL-10 favors immunologic tolerance and may increase allograft survival [11].

The antifibrotic properties of IL-10 were demonstrated in an experimental model of liver cirrhosis [10]. The use of IL-10 as a therapy in patients with HCV induced a reduction in the levels of inflammation, fibrosis score and decreased the activation of Th1 cells, showing its effect in controlling the inflammatory and fibrotic process. However, long-term administration of IL-10 favors an increase in HCV viral load due to changes in the immune response effective in eliminating the virus [12].

The objective of the present study was to quantify IL10 and TGFB1 gene expression in liver biopsy specimens from patients with HCV chronic hepatitis and to determine its roles in the pathogenesis and clinical presentation of this infection as well as in the various stages of fibrosis and liver inflammation according to the French METAVIR classification.

2. Materials and methods

Study population

The study group consisted of 53 subjects who were consecutive cases of untreated chronic carriers of HCV (n = 45) treated at the Hepatology Outpatient Clinic of the Hospital of the Fundação Santa Casa de Misericórdia do Pará (FSCMPA) in the period from 2011 to 2015. A control group (n = 8) composed of individuals who underwent conventional cholecystectomy without hepatic necroinflammatory changes and were treated at the Surgery Unit of the João...
de Barros Barreto University Hospital/UFPA was also included in this study. The patients were divided into the following three groups according to the liver disease stage as defined by the METAVIR classification [13]: without fibrosis and/or mild fibrosis (F0-F1), moderate fibrosis (F2) and severe fibrosis and/or cirrhosis (F3-F4). Normal liver samples were used as a control (CT). Further details on the patient selection criteria can be found in our previous report [14]. Patients with hepatocarcinoma (HCC), those coinfected with HIV and those being treated for hepatitis B and C were excluded from the study.

Ethical aspects
The present study was submitted to and approved by the Research Ethics Committee of Fundação Santa Casa de Misericórdia do Pará (protocol numbers 117/2009 and 684.432/2014) following the Directives and Standards Regulating Human Research (Resolution 196 of the Brazilian National Health Council). All individuals who agreed to participate in the study signed an informed consent form.

Sample collection
Liver biopsy specimens were obtained from patients with medical indications to investigate changes in the liver parenchyma. The biopsies were performed with a Tru-Cut needle and guided by ultrasound. Each sample was separated into two sections. The first section was used for a histopathological examination after hematoxylin and eosin (HE), chromotrope aniline blue (CAB), Gomori reticulin and Shikata orcein staining at the UFPA Department of Pathological Anatomy. The diagnosis was based on the French METAVIR classification; portal and periportal inflammatory activity was scored from 0 to 3, and structural changes were scored from 0 to 4. The results of the histopathological exams were obtained from the patients’ medical records, which were accessed from 2011 to 2015.

The second section of the biopsy was sent for genetic analysis at the Virology Laboratory/Biological Science Institute-ICB/UFPA and stored at -70˚C prior to use. Additionally, blood samples were collected in vacuum tubes containing EDTA as an anticoagulant, and plasma was separated by centrifugation and stored at -20˚C prior to measurement of the alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT) and viral marker levels.

RNA extraction and reverse transcription
The liver tissue fragment was stored in 500 μL of RNAlater® Tissue Collection Solution for preservation of the RNA, which was subsequently extracted using a Norgen Biotek Corporation Kit according to the manufacturer’s protocol. The RNA samples were transcribed into complementary DNA (cDNA) using a High-Capacity cDNA Reverse Transcription Kit (without inhibitor) (Applied Biosystems, USA) as previously described [13].

Quantitative real-time PCR (qPCR)
qPCR was performed in 96-well plates using TaqMan™ (Applied Biosystems, USA) on a StepOnePlus system (Life Technologies, Carlsbad, CA, USA). For both the patient and control groups, IL10 (Hs00174086_m1), TGFBI (Hs00171257_m1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs02758991_g1) gene expression assays (P/N 4326317E, Life Technologies, CA, USA) were performed in separate wells (singleplex assays). The primers were purchased from Life Technologies (Carlsbad, CA, USA). The relative expression of each gene was determined as previously described [13].
Measurement of the plasma IL-10 levels

The plasma IL-10 levels were measured using a Bio-Plex ProTM Human Cytokine 27-Plex Kit (Bio-Rad, CA, USA) and read on a LUMINEX 200 system following the manufacturer’s protocols.

Dosage of liver enzymes

The measurement of ALT, AST and GGT enzymes was carried out by colorimetric enzymatic reaction with automated methodology, using the Architect-Abbott c800 equipment (Chicago, Illinois, USA), following instructions provided by the manufacturer.

Plasma viral load

Plasma viral load was measured by real-time PCR using an Abbott RealTime HCV Amplification Reagent Kit (Abbott Park, Illinois, USA) at the Central Laboratory of the State of Pará (LACEN-PA). HCV viral load quantifications are presented as copies/mL and log10 value. The lowest detection level of HCV load was 1.08 log UI/mL, and the highest detection level was 8.00 log UI/mL.

Statistical procedures

Differences between groups were analyzed using the Kruskal-Wallis and Mann-Whitney U tests. Relationships among the variables were determined by Spearman’s correlation analysis. The level of significance was set at 5% (p value ≤ 0.05) using BioEstat 5.0 and GraphPad Prism version 6.1 software. For significant data, heatmap matrices were plotted using R 3.4.2 software with canberra and ward.D as the distance and cluster methods, respectively, depending on the options provided by the software.

Biomarker networks were obtained to evaluate correlations among the biomarkers and were analyzed using Cytoscape software. The networks were constructed using edges, representing the Spearman correlation scores, which were characterized as strongly positive (r ≥ 0.68, dark solid line), moderately positive (0.36 ≤ r ≤ 0.67; light solid line) and negative (-0.37 ≥ r; dashed line).

3. Results

In the present study, the mean patient age was 48.3 years, with 21 (46.7%) patients being female and 24 (53.3%) being male. After separating the patients according to their liver disease stage, the group of patients without fibrosis or with mild fibrosis (F0-F1) comprised the largest number of individuals, followed by those with severe fibrosis and/or cirrhosis (F3-F4) and intermediate fibrosis (F2). The median ALT, AST and GGT concentrations were elevated in all stages of the disease and were within normal values only in the control group. Inflammation (A2) and ALT levels were highest in the group with intermediate fibrosis (F2), and AST and GGT levels were highest in the group with severe fibrosis and/or cirrhosis (F3-F4) (Tables 1 and 2).

Intrahepatic and plasma IL-10 levels were significantly higher in the control group (CT) (median mRNA: 1.84; IQR: 1.55 and median plasma concentration: 14.21; IQR: 2.480) than in the group with chronic hepatitis C (median mRNA: 0.24; IQR: 0.300 and median plasma concentration: 7.95; IQR: 10.050) (p<0.0001 and p = 0.0004, respectively; Fig 1A and 1D). Regarding the disease stage, the cytokine expression levels were highest in patients with fibrosis scores F0-F1 (median: 0.365; IQR: 0.285) and decreased significantly (p = 0.0435 and p = 0.0002) as liver disease progressed to moderate fibrosis (F2) (median: 0.255; IQR: 0.1100) and severe
fibrosis and cirrhosis (F3-F4) (median: 0.210; IQR: 0.123) (p<0.0001 compared to the control group; Fig 1B). The same trend was observed for inflammation, in which the patients without liver inflammation (A0) had significantly higher IL10 expression levels (median: 0.6050; IQR: 0.688) than the patients with mild (A1) (median: 0.2900; IQR: 0.170) and moderate (A2) (median: 0.1900; IQR: 0.070) inflammatory activity (A0 compared to A1 and A2, p = 0.0043 and p = 0.0003, respectively; and A1 compared to A2 and the control, p = 0.0262 and p = 0.0013, respectively; Fig 1C). The association between the serum IL-10 levels and META-VIR scores were the same [F0-F1 (median: 11.27; IQR: 7.666), F2 (median: 7.980; IQR: 4.151) and F3-F4 (median: 7.710; IQR: 5.560)], but the differences were not significant; however, when the F0-F1 group was compared to the control group, the difference was significant (p = 0.0269; Fig 1E). Concerning inflammatory activity, the plasma concentrations were significantly higher in the group without inflammation (A0) (median: 13.86; IQR: 3.77) than in the groups with inflammation [A1 (median: 9.516; IQR: 3.76) and A2 (median: 8.995; IQR: 7.252)] (p = 0.0061 and p = 0.0100, respectively). However, the difference between the group without inflammation (A0) and the control group was not significantly different (p = 0.9754; Fig 1F).

When patients were grouped according to viral load (log10) (i.e., fibrosis score), the highest median was observed in patients in the intermediate stage of liver disease (F2) (median: 6.015; IQR: 0.527), followed by those in stages F3-F4 (median: 5.780; IQR: 0.797) and stages F0-F1 (median: 5.290; IQR: 1.551), although the difference was not statistically significant (Fig 2A). When grouped according to necroinflammatory activity, the mean plasma viral load was higher in patients in group A2 (median: 5.785; IQR: 0.715), followed by those in groups A1 (median: 5.625; IQR: 1.064) and A0 (median: 5.357; IQR: 1.749), although the difference was not statistically significant (Fig 2B).

| Variable | Chronic hepatitis C | Normal control |
|----------|---------------------|----------------|
| Subjects (n) | 45 | 8 |
| Gender (F/M) (%) | 21/24 (46.7/53.3) | 4/4 (50.0/50.0) |
| Age (mean) | 47.8 | 39.8 |
| ALT (IU/L) Median | 79.0 | 27.5 |
| AST (IU/L) Median | 66.3 | 21.0 |
| GGT (IU/L) Median | 65.9 | 29.0 |
| Fibrosis Scores<sup>a</sup> | | |
| F0 (%) | 6 (13.3) | 8 (100.0) |
| F1 (%) | 20 (44.4) | - |
| F2 (%) | 8 (17.8) | - |
| F3 (%) | 5 (11.2) | - |
| F4 (%) | 6 (13.3) | - |
| Level of Inflammation<sup>b</sup> | | |
| A0 (%) | 6 (13.3) | 8 (100.0) |
| A1 (%) | 24 (53.3) | - |
| A2 (%) | 15 (33.4) | - |

ALT: Alanine aminotransferase (ref.: 14 to 55 IU/L); AST: Aspartate aminotransferase (ref.: 14 to 32 IU/L); GGT: Gamma-glutamyl transferase (ref.: < 50 IU/L)
<sup>a</sup>Fibrosis scores: F0, absence of fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis with rare septa; F3, numerous septa without cirrhosis; and F4, cirrhosis.
<sup>b</sup>Inflammatory activity: A0, absent; A1, minimum; and A2, moderate.

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The heatmap (Fig 3A) illustrates the ability of IL10 expression to group patients according to liver disease progression and the plasma concentrations of liver enzymes. Fig 3A clearly shows increased expression patterns of this cytokine clustered in individuals without hepatic disease (CT), those with low fibrosis scores (F0-F1) and those with low plasma ALT, AST, GGT concentrations. Conversely, low IL10 expression was able to group patients with the highest fibrosis scores and the highest concentrations of the enzyme markers. Fig 3B shows the same clustering trend of IL10 expression relative to the liver inflammation levels and biochemical markers. Fig 3C shows that viral replication was not directly associated with intrahepatic IL10 expression in the chronic stages of liver disease; however, we observed small clusters grouping patients with increased viral loads in the intermediate and final stages of liver disease, although the clustering ability of IL10 expression relative to the viral load was not evident.

An interaction network between the study variables was constructed through Spearman’s correlations. We observed a negative association between IL10 expression and plasma ALT, AST and GGT concentrations at all stages of chronic liver disease. However, we also observed an increase in liver enzymes in the intermediary stage of liver fibrosis (F2) that was strongly associated with viral load. This association was weak in the initial stages, whereas in the final stages, only AST showed an association with the viral load levels. In the F0-F1 stages, a strong positive association was found between increased intrahepatic IL10 mRNA expression and an increased viral load. However, in the intermediate stage (F2) and in the late stages (F3-F4), a negative association was found between an increased viral load and decreased intrahepatic IL10 mRNA expression (Fig 4).

Fig 5 shows significantly higher TGFBI expression in the HCV group (median mRNA: 12.901; IQR: 9.070) than in the CT group (median mRNA: 3.791; IQR: 4.237; p<0.0001; Fig 5A). According to the disease stage, the lowest levels of TGFBI expression were observed in patients with a fibrosis score of F0-F1 (median mRNA: 11.410; IQR: 5.255), with a significant increase in gene expression (p = 0.0178 and p = 0.0432) as the liver disease evolved to F2 (median mRNA: 17.910; IQR: 32.75) and F3-F4 (median mRNA: 17.390; IQR: 17.730; Fig 5C). The same trend was observed with respect to necroinflammatory activity, in which TGFBI expression was significantly lower in patients without liver inflammation (A0) (median mRNA: 8.990; IQR: 4.860) than in those with mild (A1) (median mRNA: 12.05; IQR: 15.520).
and moderate (A2) (median mRNA: 12.712; IQR: 7.031) necroinflammatory activity ($p = 0.0199$ and $p = 0.0177$, respectively; Fig 5B). Fig 5D shows a significant negative correlation ($r = -0.4816$ and $p = 0.0008$) between intrahepatic $\text{IL10}$ and $\text{TGFB1}$ mRNA levels.

Fig 1. $\text{IL10}$ mRNA and plasma IL-10 levels according to the clinical condition of the liver. A-C: Intrahepatic $\text{IL10}$ expression between the HCV and control (CT) patients with different fibrosis scores and with different necroinflammatory activity levels (A: Mann-Whitney test; B and C: Kruskal-Wallis test). D-F: Plasma IL-10 concentrations between HCV and CT patients with different fibrosis scores and be with different necroinflammatory activity scores (D: Mann-Whitney test; E and F: Kruskal-Wallis test).

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and moderate (A2) (median mRNA: 12.712; IQR: 7.031) necroinflammatory activity ($p = 0.0199$ and $p = 0.0177$, respectively; Fig 5B). Fig 5D shows a significant negative correlation ($r = -0.4816$ and $p = 0.0008$) between intrahepatic $\text{IL10}$ and $\text{TGFB1}$ mRNA levels.
4. Discussion

In the present study, we observed higher intrahepatic \textit{IL10} mRNA expression levels in the control group than in the group with chronic hepatitis. IL-10 is produced by Th2 cells, and patients with chronic HCV infection show a predominance of a Th2 response that hinders

![Fig 2: HCV viral load according to the clinical condition of the liver.](https://doi.org/10.1371/journal.pone.0241199.g002)

![Fig 3: Analysis of \textit{IL10} mRNA levels according to the clinical condition of the liver and viral load represented by heatmaps.](https://doi.org/10.1371/journal.pone.0241199.g003)
Th1-dependent viral clearance. A concomitant increase in the action of Treg cells (CD25+) is believed to increase IL-10 production, thereby suppressing the Th1 response stimulated by viral proteins [15]. In addition, physiologically, the liver has a tolerogenic environment, and this suppressive condition occurs as a result of the constitutive exposure of hepatic cells to traces of endotoxins and other bacterial lipopolysaccharide (LPS) products, among other reasons, which results in the low modulation of costimulatory molecules and IL-10 synthesis by Kupffer and sinusoidal endothelial cells [16]. We also observed the same trend in the plasma IL-10 concentrations in the control and patient groups, confirming previous results [17].

HCV patients without fibrosis and without necroinflammation had lower plasma and intra-hepatic levels of IL-10 protein and mRNA than controls, suggesting that this profile may be related to the absence of viral clearance, which is characteristic of chronic infection by HCV [15, 16].

High intrahepatic IL10 mRNA expression in the F0-F1 fibrosis and A0 and A1 inflammatory activity stages indicates an association of this cytokine with viral persistence and an immunosuppressive role in the early stages of chronic hepatitis. Specifically, the plasma IL-10 concentration was also increased, but the difference was not significant.

![Interaction networks between IL10 mRNA levels and the analyzed biomarkers.](https://doi.org/10.1371/journal.pone.0241199.g004)
The immunosuppressive mechanism of IL-10 in the liver occurs when soluble antigens that pass through the liver sinusoids are absorbed by sinusoidal endothelial cells and presented to both CD8+ and CD4+ T cells, which are induced to proliferate but are unable to maintain IL-2 and IFN-γ secretion [18]. CD8+ T cells are unable to differentiate into effector cytotoxic cells, whereas CD4+ T cells can differentiate into an anti-inflammatory IL-4 and IL-10 secretory...
phenotype [18]. The low levels of IL-10 (both plasma and mRNA) observed in the groups with fibrosis (F2 and F3-F4) and with high inflammation (A2) strongly suggest that IL-10 helps control the formation of inflammation-induced fibrosis, consistent with a previous report [17].

Additionally, previous studies have reported a protective role for IL-10 in HCV infection in relation to the progression of liver fibrosis [19]. Other studies have emphasized the protective role of IL-10 during the treatment of chronic hepatitis C infection: it reduced the severity of fibrosis in participants [12]. In other studies with animal models, the absence of IL-10 was associated with liver fibrosis [20, 21].

Intrahepatic IL10 mRNA expression seems to reflect the blood pattern of this cytokine, corroborating a previous report in which HCV patients with severe fibrosis (F3-F4) had a lower frequency of intrahepatic T cells (especially Tregs) than patients with mild hepatic fibrosis. In the same study, patients with mild liver fibrosis had higher serum levels of IL-10 than patients with severe liver disease (F3-4), demonstrating similar serum and tissue levels of cytokines in HCV patients and supporting the use of serum cytokines as biomarkers associated with the liver fibrosis score [22].

Our results showed that regarding established liver fibrosis, a score of F2 was associated with the highest levels of viral replication, followed by F3-F4. High levels of inflammation (A2) in F2 corroborate data from the literature that after immune tolerance, immune activation occurs, with increased viral replication, causing inflammation and hepatocyte necrosis, which can be reflected by the increase in liver enzymes together with the regeneration of liver cells and marked fibrous cell hyperplasia [16].

Intrahepatic IL10 mRNA expression showed a positive association with an increased plasma viral load in the early disease stages (F0-F1); however, this association was not observed in the intermediate (F2) and late (F3-F4) stages. In the F2 and F3-F4 stages, a positive correlation between viral persistence and the hepatic necroinflammation markers ALT, AST and GGT was observed, with the highest significance found in the intermediate stage of fibrosis (F2), at which point the highest levels of necroinflammatory activity (A2) were also observed. In this context, our results also showed an increase in the serum IL-10 levels in the early stages of fibrosis (F0-F1) and a decline in the final stages, with statistically significant differences. Some studies have shown that elevated IL-10 levels correlate with decreased T cell activity in patients with HCV and the inability of these cells to control viral replication, which emphasizes the important role of T cell depletion in potentiating viral persistence. This finding suggests that the outcome of the immune response for the prevention of viral persistence is not dictated by the initially high viral replication levels; in contrast, a generalized infection can be quickly contained by the maintenance of T cell activity [23]. A study of a cohort of women with persistent HCV infection revealed that CD4+ T cells secreted IFN-γ and IL-10 in response to the core protein and demonstrated that CD4+ and Treg T cells were induced against the same epitopes of the core protein during HCV infection [24].

Experimental studies in cell cultures have shown that HCV induces an increase in IL-10 in CD4+ T in lymphocyte cell lines [25]. The presence of IL-10 in the culture of naive CD8+ T cells reduced the frequency of specific cells, showing an association between the early production of IL-10 and the failure to activate specific T cells and viral persistence [26].

Our results showing a negative association between intrahepatic IL10 mRNA expression and increased ALT, AST and GGT levels with the progression of liver disease to cirrhosis corroborate reports in the literature demonstrating the antifibrotic and antifibrogenic roles of IL-10 in liver damage. This finding is in agreement with results showing increased intrahepatic IL10 mRNA expression levels in the early stage of fibrosis that disappear in advanced stages, suggesting that IL-10 released by hepatic stellate cells (HSCs) suppresses collagen production.
through a negative self-regulatory role following the induction of collagenase during the early stage of liver fibrosis. On the other hand, the lack of intrahepatic IL10 mRNA expression by HSCs in association with the induction of collagenase leads to advanced liver fibrosis [24]. Furthermore, in HCV infection, specific IL10 polymorphisms are correlated with increased susceptibility to the development of chronic infection and increased severity of hepatic disease [27].

TGF-β1 participates in different stages of liver disease progression and in the activation and differentiation of HSCs, with subsequent stimulation of extracellular matrix proteins, such as collagen and fibronectin, inducing fibrosis [1, 28]. In our study, the intrahepatic expression of TGFBI was approximately four times higher in HCV carriers than in controls; moreover, a significant increase in this gene was associated with the highest levels of necroinflammatory activity and high liver fibrosis scores, demonstrating that in chronic hepatitis, with the failure of the immune system to control viral replication, there is a persistent recruitment of mononuclear inflammatory infiltrate, leading to chronic inflammation with sustained liver damage that is modulated by several factors, including TGF-β1 and IL10 [29, 30]. Furthermore, the negative correlation between the highest levels of TGFBI mRNA expression levels and low intrahepatic IL10 mRNA expression levels in the livers of individuals with HCV demonstrates that chronically infected patients have decreased IL-10 levels with the continuous stimulation of factors that lead to the synthesis of TGF-β1, with the consequent progression of fibrosis and evolution to cirrhosis, corroborating previous studies [30, 31].

Taken together, our results demonstrate that initially, the constitutive expression of IL10 in the liver induces the tolerance of virus-specific CD8+ T cell infiltrates, decreases their proliferative capacity and results in the loss of antiviral effector functions, which are important for the decrease in immunopathogenesis at the expense of viral persistence. In addition, increased intrahepatic IL10 mRNA expression is likely to maintain HSCs in a quiescent stage by suppressing their profibrogenic function in the early disease stages. However, intrahepatic IL10 mRNA expression decreases with persistent fibrosis, probably due to the perpetuation of activated HSCs with production of the extracellular matrix and TGF-β1, which is considered a potent antimitotic and fibrogenic cytokine, thereby making liver damage irreversible [32, 33].

**Conclusion**

The inhibitory activity of IL-10 produced locally in the liver can limit and weaken the immune response, promote viral persistence and indirectly limit the fibrogenic response by controlling TGF-β1 secretion. However, a decrease in hepatic IL-10 favors an increase in persistent inflammatory infiltrate, resulting in severe fibrosis and cirrhosis.

**Supporting information**

S1 Dataset.
(PDF)

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**Author Contributions**

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References

1. Pellicoro A, Ramachandran P, Iredale J, Fallowfield J. Liver fibrosis and repair: immune regulation of wound healing in a solid organ. Nature Reviews Immunology. 2014, 14:181–194. https://doi.org/10.1038/nri3623 PMID: 24566915

2. Manigol d T, Racanelli V. T-cell regulation by CD4 regulatory T cells during hepatitis B and C virus infections: facts and controversies. Lancet Infect Dis. 2007, 7:804–813. https://doi.org/10.1016/S1473-3099(07)70289-X PMID: 18045563

3. Crispe IN, Matthew G, Ingo K, Beena J, Bradford S, Sherry W. Cellular and molecular mechanisms of liver tolerance. Immunological Reviews. 2006, 203:101–118. https://doi.org/10.1111/j.1600-065X.2006.00435.x PMID: 16972899

4. Bjernack A, Dobaczewski M, Frangogiannis NG. TGF-β signaling in fibrosis. Growth Factors. 2011, 29:196–202. https://doi.org/10.3109/08977194.2011.595714 PMID: 21740331

5. Fiorentino DF, Bond MW, Mosmann TR. Two types of mouse T-helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. Journal Experimental Medicine. 1989, 170:2081–2095. https://doi.org/10.1084/jem.170.6.2081 PMID: 2531194

6. Dumoutier L, Renaudin JC. Viral and cellular interleukin-10 (IL-10)-related cytokines: from structures to functions. European Cytokine Network. 2002, 13:5–15. PMID: 11956016

7. Moore KW, De Waal Malefyt R, Coffman RL, O'garra A. Interleukin-10 and the interleukin-10 receptor. Annual Review Immunology. 2002, 19:683–765.

8. Brooks DG, Trifilo MJ, Edelmann KH, Teyton L, Mcgavern DB, Oldstone MB. Interleukin-10 determines viral clearance or persistence in vivo. Nature Medicine. 2006, 12:1309–13. https://doi.org/10.1038/nm1492 PMID: 17041596

9. Wan S, Leclerc JL, Schmartz D, Barvais L, Huynh CH, Deviere J, et al. Hepatic release of interleukin-10 during cardiopulmonary bypass in steroid-pretreated patients. American Heart Journal. 1997, 133:335–339. https://doi.org/10.1016/s0002-8703(97)70229-1 PMID: 9060803

10. Zhang Li-Juan Wang Xiao-Zhong. Interleukin-10 and chronic liver disease World Journal Gastroenterology. 2006, 12:1681–1685. https://doi.org/10.3748/wjg.v12.i11.1681 PMID: 16586534

11. Barrat F, Cua DJ, Boonstra A, Richards DF, Crain C, Savelkoul H, et al. In vitro generation of IL-10-producing regulatory CD4+ T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 and Th2 inducing cytokines. Journal Experimental Medicine. 2002, 195:603–616. https://doi.org/10.1084/jem.20011629 PMID: 11877483

12. Nelson Dr, Tu Z, Soldevila-Pico C, Abdelmalek M, Zhu H, Xu Yl, et al. Long-term interleukin 10 therapy in chronic hepatitis C patients has a proviral and antifibrotic effect. Hepatology. 2003, 38: 859–868. https://doi.org/10.1053/jhep.2003.50427 PMID: 14512873
13. Bedossa P, Poynard T. An Algorithm for the Grading of Activity in Chronic Hepatitis C. Hepatology. 1996, 24:2. https://doi.org/10.1022/hep.510240201 PMID: 8690394

14. Amoras ESG, Gomes STM, Freitas FB, Santana BB, Ishak G, Araújo MT et al. NGF and P75NTR gene expression is associated with the hepatic fibrosis stage due to viral and non-viral causes. PLoS One. 2015, 10: e0121754. https://doi.org/10.1371/journal.pone.0121754 PMID: 25816145

15. Gramenz A, Andreone P, Loggi E, Foschi FG, Cursaro C, Margotti M, et al. Cytokine profile of peripheral blood mononuclear cells from patients with different outcomes of hepatitis C virus infection. Journal of Viral Hepatitis. 2005, 12:525–530. https://doi.org/10.1111/j.1365-2893.2005.00634.x PMID: 16108769

16. Crispe I.N. The Liver as a Lymphoid Organ. Annual Reviews Immunology. 2009, 27:147–63. https://doi.org/10.1146/annurev.immunol.021908.132629 PMID: 19302037

17. Souza-Cruz S, Victória MB, Tarragô AM, Costa AG, Pimentel GPD, Pires EF, et al. Liver and blood cytokine microenvironment in HCV patients is associated to liver fibrosis score: a proinflammatory cytokine ensemble orchestrated by TNF and tuned by IL-10. BMC Microbiology. 2016, 16:3 https://doi.org/10.1186/s12866-015-0610-6 PMID: 26742960

18. Knolle PA, Schmitt E, Jin S, Germann T, Duchmann R, Hegenbarth S, et al. Induction of cytokine production in naïveCD4+ T cells by antigen-presenting murine liver sinusoidal endothelial cells but failure to induce differentiation toward TH1 cells. Gastroenterology. 1999, 116:1428–1440. https://doi.org/10.1016/s0016-5085(99)70508-1 PMID: 10348827

19. Aroucha DC, do Carmo RF, Moura P, Silva JL, Vasconcelos LR, Cavalcanti MS. High tumor necrosis factor-α/interleukin-10 ratio is associated with hepatocellular carcinoma in patients with chronic hepatitis C. Cytokine. 2013, 62:421–5. https://doi.org/10.1016/j.cytokine.2013.03.024 PMID: 23602201

20. Louis H, Le MO, Peny MO, Quertinmont E, Fokin D, Goldman M, et al. Production and role of interleukin-10 in concanavalin A–induced hepatitis in mice. Hepatol. 1997; 25:1382–9. https://doi.org/10.1002/hep.510250614 PMID: 9185757

21. Thompson K, Maltby J, Failowli Eld J, Mcaulay M, Millward-Adler H, Sheron N. Interleukin-10 expression and function in experimental murine liver inflammation and fibrosis. Hepatology. 1998, 28:1597–1606. https://doi.org/10.1002/hep.510280620 PMID: 9828224

22. de Souza-Cruz S, Victória MB., Tarragô AM et al. Liver and blood cytokine microenvironment in HCV patients is associated to liver fibrosis score: a proinflammatory cytokine ensemble orchestrated by TNF and tuned by IL-10. BMC Microbiol. 2016, 16:3 https://doi.org/10.1186/s12866-015-0610-6 PMID: 26742960

23. Rigopoulou EI, Abbott WHG, Haigh P, Naoumov NV. Blocking of interleukin-10 receptor—a novel approach to stimulate T-helper cell type 1 responses to hepatitis C virus. Clinical Immunology. 2005, 117:57–64. https://doi.org/10.1016/j.clim.2005.06.003 PMID: 16006191

24. Macdonald AJ, Duffy M, Brady MT, Mckierman S, Hall W, Hegarty J, et al. CD4 T Helper Type 1 and Regulatory T Cells Induced Against the Same Epitopes on the Core Protein in Hepatitis C Virus–Infected Persons. The Journal of Infectious Disease. 2002, 185:720–727. https://doi.org/10.1086/339340 PMID: 11920289

25. Delpuech O, Buffello-Le Guillou DB, Rubinstein E, Féray C, Petit MA. The hepatitis C virus (HCV) induces a long-term increase in interleukin-10 production by human CD4+ T cells (H9). Eur Cytokine Netw. 2001, 12:69–77. PMID: 11282549

26. Niesen E, Schmidt J, Fleckten T, Thimme R. Suppressive effect of interleukin 10 on priming of naive hepatocellular carcinoma in patients with chronic hepatitis C. Cytokine. 2013, 62:421–5. https://doi.org/10.1016/j.cytokine.2013.03.024 PMID: 23602201

27. Macdonald AJ, Duffy M, Brady MT, Mckierman S, Hall W, Hegarty J, et al. CD4 T Helper Type 1 and Regulatory T Cells Induced Against the Same Epitopes on the Core Protein in Hepatitis C Virus–Infected Persons. The Journal of Infectious Disease. 2002, 185:720–727. https://doi.org/10.1086/339340 PMID: 11920289

28. Delpeuch O, Buffello-Le Guillou DB, Rubinstein E, Féray C, Petit MA. The hepatitis C virus (HCV) induces a long-term increase in interleukin-10 production by human CD4+ T cells (H9). Eur Cytokine Netw. 2001, 12:69–77. PMID: 11282549

29. Wang SC, Ohata M, Schrum L, Rippe RA, Tsukamoto H. Expression of interleukin-10 by in vitro and in vivo activated hepatic stellate cells. The Journal of Biological Chemistry. 1998, 273:302–308. https://doi.org/10.1074/jbc.273.1.302 PMID: 9417090

30. Persico M, Capasso M, Persico E. Interleukin-10–1082 GG polymorphism influences the occurrence and the clinical characteristics of hepatitis C virus infection. Journal of Hepatology. 2006, 45:779–785. https://doi.org/10.1016/j.jhep.2006.07.026 PMID: 17049666

31. Pinzani M, Marra F. Cytokine receptors and signaling in hepatic stellate cells. Seminary Liver Disease. 2001, 21:397–416. https://doi.org/10.1067/s1537-2905(01-1755-4) PMID: 11586468

32. Larrubia JR, Benito-Martinez S, Miquel-Plaza J, Sanz-de-Villalobos E, Gonzalez-Mateos F, Parra T. Cytokines—their pathogenic and therapeutic role in chronic viral hepatitis. Revista Española de Enfermedades Digestivas. 2009 1015:343–351. https://doi.org/10.4321/s1130-0108/2009006500060 PMID: 19527080

33. Chusri P, Kumthip K, Jian Hong, Zhu C, Duan X, Jilg N, et al. HCV induces transforming growth factor β1 through activation of endoplasmic reticulum stress and the unfolded protein response. Sci. Rep. 2016, 6:2487. https://doi.org/10.1038/srep2487 PMID: 26927933
32. Malhi H, Guicciardi MM, Gores GJ. Hepatocyte Death: A Clear and Present Danger. Physiological Reviews. 2010, 90:1165–1194. https://doi.org/10.1152/physrev.00061.2009 PMID: 20664081

33. Louis H, Le Moine O, Goldman M, Devière J. Modulation of liver injury by interleukin-10. Acta Gastroenterology Belgic. 2003, 66:7–14. PMID: 12812143