Research Article

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Cirrhosis

Type I interferon signaling in systemic immune cells from patients with alcoholic cirrhosis and its association with outcome☆

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Background & Aims: In immune cells, constitutively and acutely produced type I interferons (IFNs) engage autocrine/paracrine signaling pathways to induce IFN-stimulated genes (ISGs). Enhanced activity of IFN signaling pathways can cause excessive inflammation and tissue damage. We aimed to investigate ISG expression in systemic immune cells from patients with decompensated alcoholic cirrhosis, and its association with outcome.

Methods: Peripheral blood mononuclear cells (PBMCs) from patients and heathy subjects were stimulated or not with lipopolysaccharide (LPS, an IFN inducer) or increasing concentrations of IFN-β. The expression of 48 ISGs and ten "non-ISG" inflammatory cytokines were analyzed using RT-qPCR.

Results: We developed an 8-ISG signature (IFN score) assessing ISG expression. LPS-stimulated ISG induction was significantly lower in PBMCs from patients with cirrhosis compared to healthy controls. Non-ISGs, however, showed higher induction. Lower induction of ISGs by LPS was not due to decreased IFN production by cirrhotic PBMCs or neutralization of secreted IFN, but a defective PBMC response to IFN. This defect was at least in part due to decreased constitutive ISG expression. Patients with the higher baseline IFN scores and ISG levels had the higher risk of death. At baseline, "non-ISG" cytokines did not correlate with outcome.

Conclusions: PBMCs from patients with decompensated alcoholic cirrhosis exhibit downregulated ISG expression, both constitutively and after an acute stimulus. Our finding that higher baseline PBMC ISG expression was associated with higher risk of death, suggests that constitutive ISG expression in systemic immune cells contributes to the prognosis of alcoholic cirrhosis.

Lay summary: Enhanced activity of IFN signaling pathways can cause excessive inflammation and tissue damage. Here we show that peripheral blood mononuclear cells (PBMCs) from patients with alcoholic cirrhosis exhibit a defect in interferon-stimulated genes (ISGs). We found that higher baseline ISG expression in PBMCs was associated with higher risk of death, revealing a probable contribution of ISG expression in immune cells to the outcome of alcoholic cirrhosis.

Keywords: Interferons; Innate immune response; Lipopolysaccharide; Liver cirrhosis; Prognosis; Leukocytes, mononuclear; Inflammation; Cytokines; Liver cirrhosis, alcoholic.

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Introduction

Type I interferons (IFNs) are a family of cytokines that play a crucial role in the immune response to viral pathogens [1]. They mediate virus detection by intracellular or extracellular sensors and the activation of antiviral mechanisms, via an autocrine/paracrine signaling (Fig. 1) [2]. IFNs engage canonical signaling pathways through a common heterodimeric receptor (IFNAR1/

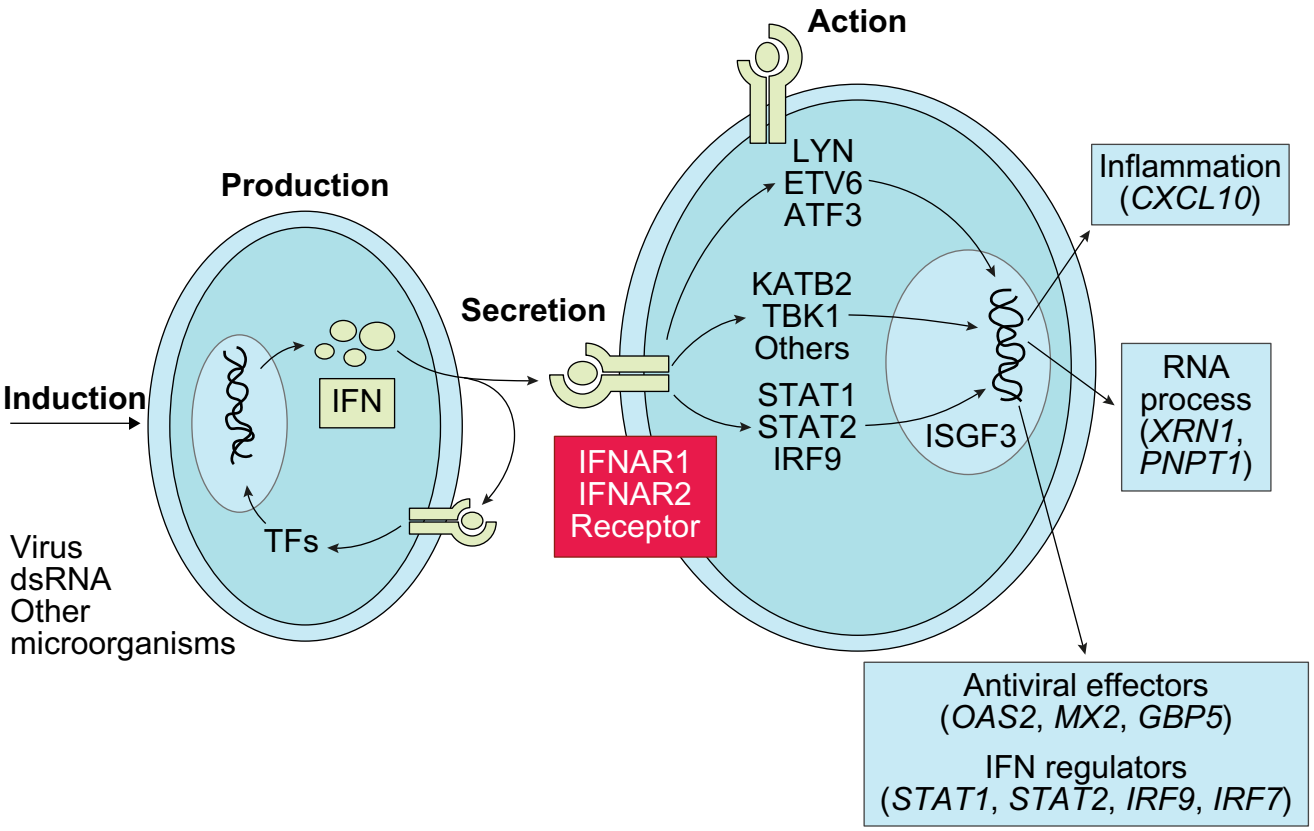


Fig. 1. Autocrine/paracrine signaling of IFN leading to ISG induction. See Supplementary legend to Fig. 1. (This figure appears in colour on the web.)

IFNAR2) [3]. Receptor subunits are associated with the kinases JAK1 and TYK2, and activation of these kinases results in phosphorylation of STAT1 and STAT2, which then associate with IFN regulatory factor (IRF)9 to form the IFN-stimulated gene factor (ISGF)3 complex activating the transcription of IFN-stimulated genes (ISGs) [4,5]. ISGs are involved in innate antiviral immunity and include members of the interferon induced protein with tetratricopeptide repeats (*IFIT*), myxovirus resistance (*MX*), 2′-5′ -oligoadenylate synthetase (*OAS*) and guanylate-binding protein (*GBP*) families and some *IFNs*, themselves [2,5]. In addition, IFNs engage non-canonical, ISGF3-independent signaling pathways to elicit ISGs that mediate inflammation C-X-C motif ligand 10 (*CXCL10*) or RNA processing 5′-3′ exoribonuclease 1 (*XRN1*) [5].

The Gram-negative bacteria byproduct lipopolysaccharide (LPS) is a pathogen-associated molecular pattern (PAMP) molecule, which is recognized by Toll-like receptor (TLR) 4, a pathogen recognition receptor (PRR) expressed at the surface of innate immune cells (e.g., monocytes/macrophages) [6]. In these cells, TLR4 engagement stimulates different signaling pathways to induce a battery of genes encoding inflammatory molecules, including prototypical pro-inflammatory cytokines (tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1β) and chemokines (CXCL-8), and anti-inflammatory cytokines (IL-10) [6]. Intriguingly, in immune cells from mice [7–9] and humans [10–12], TLR4 engagement also induces IFNs [11–13] stimulates their secretion via a trans-Golgi process [14] and induces a large number of canonical and non-canonical ISGs [10–12]. There is evidence that upregulated IFN activity can be detrimental at the organismal level. For example, in mouse models of LPS-induced shock, IFN signaling contributes to the excessive immune response of the host that cause collateral tissue damage (a process called immunopathology) (Supplementary material) [15]. Moreover, human interferonopathies (such as Aicardi–Goutières syndrome) are Mendelian disorders in which a constitutive upregulation of IFN activity is associated with tissue damage (Supplementary Table 1). Finally, several auto-inflammatory diseases (e.g., systemic lupus erythematosus) are associated with features of increased IFN activity and tissue injury [5].

Patients with cirrhosis and Gram-negative infection are highly susceptible to severe sepsis and septic shock, which may be explained by an overproduction of pro-inflammatory cytokines and chemokines at the early stage of infection. Consistent with this hypothesis, the *ex vivo* LPS-induced production of TNF-α [16], and IL-6 [10] is higher and that of IL-10 is lower in peripheral blood mononuclear cells (PBMCs) or monocytes from patients with decompensated cirrhosis than in those from healthy subjects. Interestingly, a recent study on a small series of patients indicated that PBMCs from patients with decompensated cirrhosis may exhibit a defect in *ex vivo* LPS-induced ISGs [10]; but the mechanisms underlying the defect in ISG induction were not assessed [10]. Therefore, to address these questions, we isolated PBMCs in a large number of patients with decompensated alcoholic cirrhosis and healthy subjects, and investigated ISG expression in unstimulated and LPS-stimulated cells. In addition, experiments were performed to stimulate PBMCs with other IFN-activating PAMPs or with IFN-β (an IFN). We found that PBMCs from patients with decompensated alcoholic cirrhosis exhibited an *ex vivo* defect in ISG expression constitutively and

following stimulation with different agonists. Moreover, the constitutive ISG expression levels were related to patients' outcome; the higher the baseline ISG expression, the higher the risk of death.

Patients and methods

Patients

Blood samples were obtained with written informed consent from 101 hospitalized patients with stable decompensated biopsy-proven cirrhosis (Supplementary materials and methods and Supplementary Table 2). The study was approved by the local ethics committees.

Gene expression and signaling

PBMCs or monocytes were isolated and cultured as previously described [10]; HuH7 cells were also used. Study protocols are detailed in the Supplementary materials and methods and Supplementary Table 2; mRNA expression was monitored using RT-qPCR (primers in Supplementary Table 3). Immunostaining was used as previously described [17].

Statistical analysis

See Supplementary materials and methods.

Results

Defective ex vivo ISG induction in LPS-stimulated cirrhotic PBMCs

In a previous study [10], using expression microarray, we found a subgroup of 652 genes whose LPS-induced expression was significantly lower in PBMCs from four patients with decompensated cirrhosis than in those from four healthy subjects. Among these 652 genes, a large number enriched the gene ontology term "Response to virus"[10], suggesting defective ISG induction in cirrhotic cells. In preparation of the present study, we queried the interferome public database (http://interferome.its.monash.edu.au) with the 652 differentially expressed genes and found that 163 were IFN-induced ISGs (Supplementary Table 5A).

Next, we decided to validate results of expression microarray using RT-qPCR in PBMCs from a large cohort of newly recruited patients with decompensated alcoholic cirrhosis and healthy controls. PBMCs were left unstimulated or were stimulated with LPS for 4 h; RNA was then extracted. For RT-qPCR experiments, we selected 48 genes: 47 among the 163 IFN-induced ISGs previously found less expressed in LPS-stimulated cirrhotic PBMCs [10] and *IFNB1*. The 48 ISGs were selected according to their dependence on signaling pathways. Twenty-eight were chosen because: a) they are representative of the larger cluster of IFN-inducible ISGs, which is characterized by its dependence on the activation of canonical IFN signaling pathways resulting in ISGF3 complex formation; and b) they are major antiviral effectors (e.g., *DDX58* [encoding the cytosolic retinoic acid-inducible gene I (RIG-I)-like receptor (RLR) RIG-I], *IFIH1* [encoding the RLR MDA5]) or IFN regulators (e.g., *IRF1* [encoding a transcription factor], *USP18* [a strong negative regulator of IFN signaling]) (Supplementary Table 5B) [5]. Eight other ISGs were selected because they are induced by non-canonical IFN signaling pathways (Supplementary Table 5B) and are involved in inflammation (e.g., *CXCL10*) or RNA processing (e.g., *XRN1*) [5,18]. Eleven ISGs are representative of the set of ISGs with unclear mechanisms of induction (e.g., indoleamine 2,3-dioxygenase [*IDO1*]; Supplementary Table 5B) [5]. The last selected ISG was *IFNB1*, encoding IFN-β a prototypical IFN (Supplementary Table 5B) [5]. The 48 ISGs were also selected because, despite apparent redundancy, most have unique functional characteristics (Supplementary Table 5B). For example, *DDX58*, *IFIH1*, *TLR3* which are all PRRs that recognize double-stranded RNA (dsRNA), are in fact distinct in that, two (*DDX58*, *IFIH1*) encode RLRs located in the cytosol and the third one (*TLR3*) encodes an endosomal PRR. Moreover, among RLRs, one detects short dsRNA (*DDX58*) and the other long dsRNA (*IFIH1*). The molecules that detect cytosolic DNA (*AIM2*, *ZBP1*, *IFI16*, and *MB21D1*) are another example. Indeed, they differ each other by the signaling pathways they activate: *AIM2* product activates the inflammasome-dependent production of IL-1β; *ZBP1*, *IFI16*, and *MB21D1* gene products activate STING-dependent IFN production, but via distinct mechanisms that confer specificity. Finally, the *MX* and *GBP* families both encode GTPases but *MX* gene products have a strong antiviral activity while several *GBP*s encode proteins involved in the defense against intracellular bacteria. In addition, we investigated five "non-ISG" cytokines (*IFNG* [a type II interferon], *IL10*, *IL6*, *IL1B*, and *TNF*), and five "non-ISG" chemokines (*CXCL1*, *CXCL2*, *CXCL3*, *CXCL5*, *CXCL8*). The term "cirrhotic PBMCs" will be used hereafter instead of "PBMCs from patients with decompensated alcoholic cirrhosis". In the present study, the expression of each ISG, in unstimulated cirrhotic PBMCs, LPS-stimulated cirrhotic and healthy PBMCs was normalized according to the expression of the gene in unstimulated healthy PBMCs (see Supplementary materials and methods section).

The 48 ISGs were significantly induced by LPS in healthy PBMCs (Fig. 2A; Supplementary Table 5C). In LPS-stimulated cirrhotic PBMCs compared to corresponding healthy cells, 35 out of the 48 ISGs (72.9%) had weaker induction in cirrhotic than in healthy PBMCs (Fig. 2A and B; Supplementary Table 5C). Defective ISGs were targets of either canonical or non-canonical IFN signaling pathways (Fig. 2A; Supplementary Tables 5B and 5C). *IFNG*, *IL10*, *IL6*, *IL1B*, and *CXCL8* had similar levels of induction in both groups. *TNF*, *CXCL2*, *CXCL3* and *CXCL5* had significantly higher LPS-induced expression in cirrhotic PBMCs (Fig. 2C and Supplementary Table 5C). Our results confirm the existence of defective ISG induction in LPS-stimulated cirrhotic PBMCs. The defect was already present after 1 h LPS (Fig. 2D), and was also shown, albeit to a lesser extent, in LPS-stimulated monocytes (Supplementary results; Supplementary Fig. 1).

Development of an IFN score

Since there are challenges with directly assessing IFN production [19], several studies have quantified IFN levels by measuring an ISG signature [20–24]. We developed an 8-ISG signature (IFN score) including *MX2*, *OAS2*, *DDX58*, *IFIT1*, *IFIH1*, *TRIM22*, *GBP4*, and *CXCL10*. These ISGs were chosen first because of their strong induction by LPS in healthy PBMCs and their defective induction in LPS-stimulated cirrhotic PBMCs (Supplementary Table 5C). Then, we wished to have a score that takes into account the difference in the number of genes that compose the clusters of IFN-inducible ISGs (Supplementary Table 5B) [5]: 6 genes (*MX2*, *OAS2*, *DDX58*, *IFIT1*, *IFIH1*, and *GBP4*) are representative of the largest cluster of canonical ISGs; the two remaining ISGs are representative of smaller clusters (*CXCL10*,

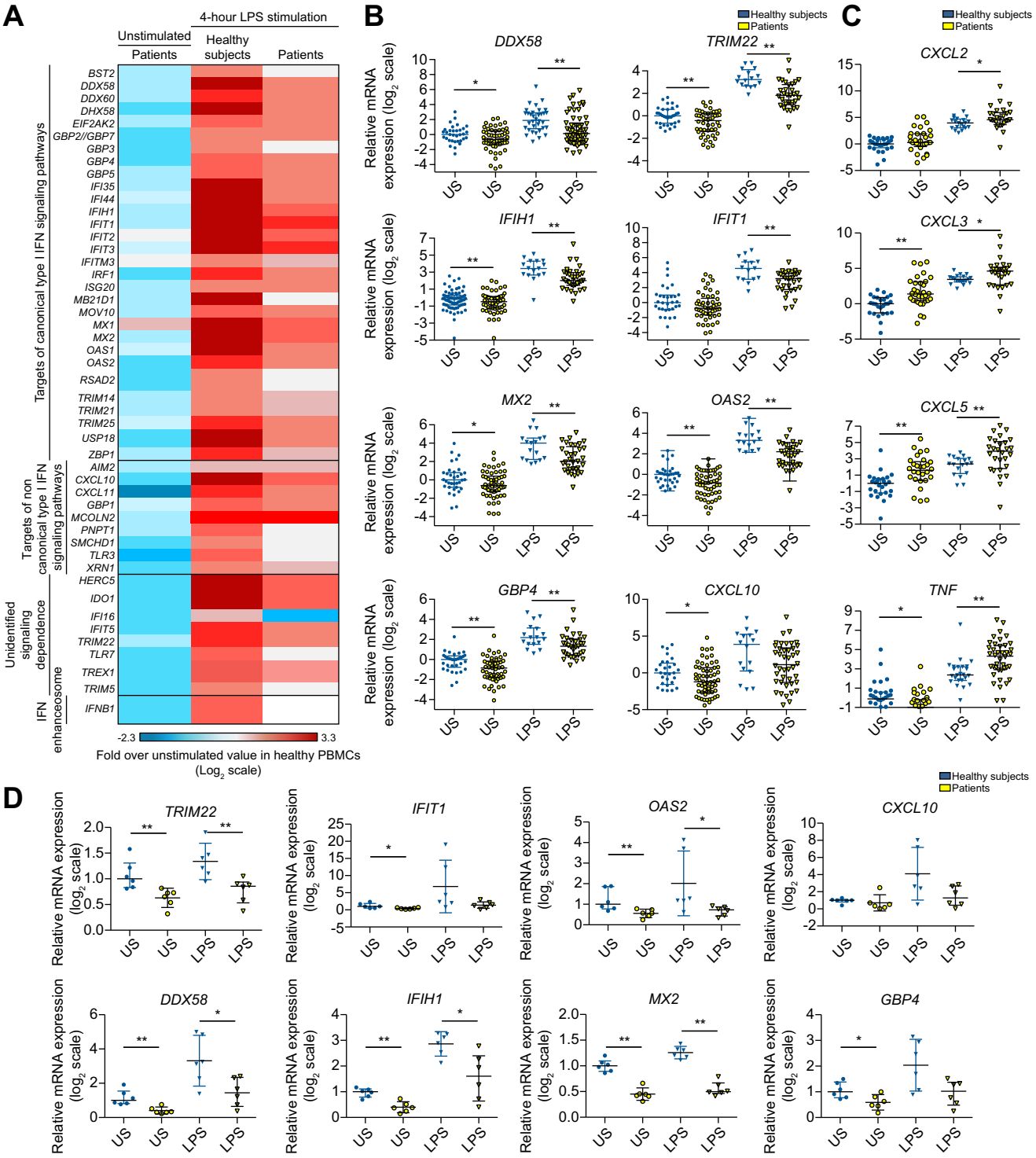


Fig. 2. Defective *ex vivo* LPS-elicited ISG induction in peripheral blood mononuclear cells (PBMCs) and monocytes from patients with cirrhosis. (A) Forty-eight ISGs in unstimulated cirrhotic PBMCs, and cirrhotic and healthy PBMCs stimulated with LPS (1 μg/ml) for 4 h (n = 37 and n = 17, respectively). (B) Representative ISGs from (A). (C) "Non-ISG" cytokines in cirrhotic and healthy PBMCs stimulated or not with LPS. (D) ISGs in cirrhotic and healthy PBMCs after 1 h LPS (n = 6 in both groups). Values are median (IQR). US denotes unstimulated conditions. $^{*}p$ <0.05, $^{**}p$ <0.01 (Mann-Whitney test). (This figure appears in colour on the web.)

TRIM22). Finally, score components were selected according to their functions; there are major sensors of viral nucleic acids virus sensors (such as *DDX58*, *IFIH1*, *IFIT1* and *OAS2*), crucial antimicrobial GTPases (*MX2*, *GBP4*), prototypical inflammatory chemokine (*CXCL10*) or molecules known to induce ubiquitination of viral proteins (*TRIM22*). As expected, the IFN score was significantly lower in cirrhotic LPS-stimulated PBMCs than in corresponding healthy PBMCs (Fig. 3A).

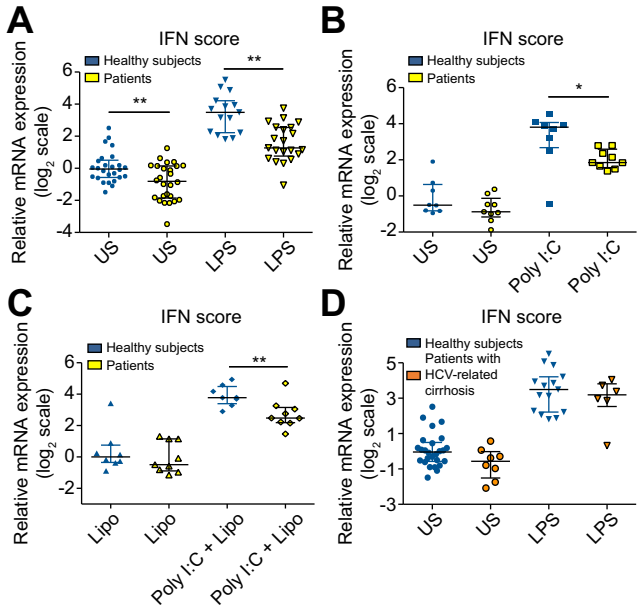


Fig. 3. 8-ISG signature (IFN score) capturing ISG PBMC expression. (A) PBMCs after 4 h LPS. (B) PBMCs after 4 h poly (I:C) (10 μg/ml). (C) PBMCs after 4 h poly (I:C) plus lipofectamine. Results shown in (B) and (C) were obtained in nine patients and eight healthy subjects. Patients in (A), (B) and (C) had alcoholic cirrhosis. (D) IFN score calculated in LPS-stimulated PBMCs from patients with non-alcoholic cirrhosis (n = 10). Values are median (IQR). US denotes unstimulated conditions. $^{*}p$ <0.05, $^{**}p$ <0.01 (Mann-Whitney test). (This figure appears in colour on the web.)

Next, we asked whether the IFN score captured PBMC ISG induction obtained with IFN-activating PAMPs other than LPS. Microbial dsRNAs are PAMPs detected by TLR3 (when dsRNAs are in endolysosome compartment) or by RLRs (when dsRNAs are in the cytosol). dsRNA recognition by TLR3 or RLRs results in IFN production and subsequent ISG induction [25,26]. Our set of 48 ISGs was measured in healthy and cirrhotic PBMCs that were left unstimulated or stimulated with either polyinosinic: polycytidylic acid (poly (I:C); a synthetic dsRNA, detected by TLR3) [26] or poly (I:C) in complex with lipofectamine (this complex being detected by RLRs) [25]. In healthy PBMCs, the IFN score was significantly higher in PBMCs stimulated with poly (I:C) alone or combined with lipofectamine than in corresponding unstimulated PBMCs (Fig. 3B and C). The IFN scores calculated in cirrhotic PBMCs stimulated with poly (I:C) alone or combined with lipofectamine were significantly lower than the corresponding IFN scores in healthy PBMCs stimulated with these PAMPs (Fig. 3B and C). Cirrhotic PBMCs had defective induction of 4 IFN score components (*MX2*, *OAS2*, *DDX58*, *TRIM22*) with either LPS, poly (I:C), or poly (I:C) plus lipofectamine (Supplementary Table 5C, 6; Supplementary Fig. 2).

The defect in ISG induction is more marked with LPS than with other IFN-activating PAMPs

Next, we analyzed in detail the responses to poly (I:C) alone or combined with lipofectamine and compared these responses to those elicited by LPS. In healthy PBMCs, poly (I:C) alone or combined with lipofectamine significantly induced 47 out of the 48 LPS-inducible ISGs, with only minor differences in the induction profile between the two stimuli (Supplementary Table 6). In cirrhotic PBMCs, there was a defect in ISG induction with poly (I:C) alone or combined with lipofectamine but the defect was less extensive with these PAMPs (11/48 or 13/48) than with LPS (35/48) (Supplementary Fig. 2). Seventeen ISGs were specifically defective in LPS-stimulated cirrhotic PBMC; none of the 13 "normally" induced ISG in LPS-stimulated cirrhotic PBMCs had defective induction after stimulation with other "non-LPS" PAMPs (Supplementary Table 6; Supplementary Fig. 2). There were six defective ISGs shared by the three PAMPs, five defective ISGs shared by LPS and poly (I:C) only; seven defective ISGs shared by LPS and poly (I:C) plus lipofectamine only (Supplementary Fig. 2).

No defect in LPS-elicited ISG in non-alcoholic cirrhosis

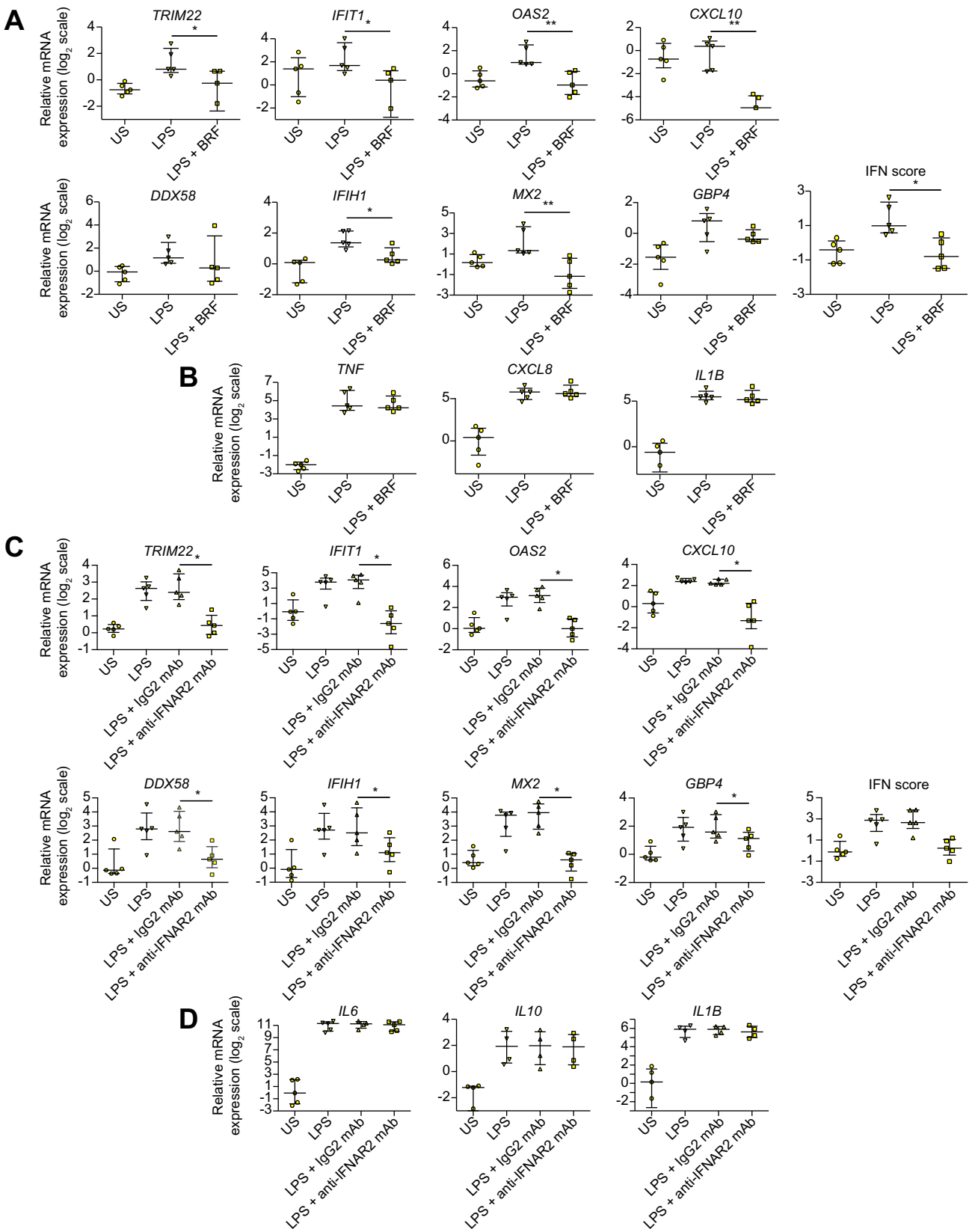
PBMCs from eight patients with decompensated hepatitis C virus (HCV)-related cirrhosis (Supplementary Table 7) were left unstimulated or stimulated with LPS for 4 h. The IFN score did not significantly differ between PBMCs from patients with HCV-related cirrhosis and corresponding healthy PBMCs (Fig. 3D), suggesting that the defect in ISG induction is related to the alcoholic etiology of cirrhosis rather than its severity. Indeed, there was no difference in the severity of liver disease between patients with alcoholic cirrhosis and those with HCV-related cirrhosis. Moreover, among alcoholics, there was no significant correlation between the LPS-induced IFN score and the model of end-stage liver disease (MELD) score ($r_s = -0.18$, $p = 0.33$).

The LPS-elicited ISG induction is mediated by interferon alpha receptor

We investigated the effects of brefeldin A (GolgiStop), a blocker of protein secretion [14,27], and found a marked decrease in LPS-induced ISG expression in cirrhotic PBMCs pre-treated with brefeldin A (Fig. 4A), confirming that protein secretion is a prerequisite for LPS-elicited ISG induction. LPS induction of pro-inflammatory *IL1B*, *CXCL8* and *TNF* was not affected by brefeldin A showing that the induction of these cytokines does not involve a brefeldin A-sensitive secretion pathway (Fig. 4B). We then studied IFNAR mediation in LPS-elicited ISG induction. Since IFNAR2 inhibition is known to render the heterodimer IFNAR unresponsive to IFNs [28], we investigated the effects of an IFNAR2 antagonist on the induction of a set of ISGs and "non-ISG" genes in LPS-stimulated cirrhotic and healthy PBMCs. The IFNAR2 antagonist inhibited LPS-induced expression in 80% of measured ISGs (Fig. 4C; Supplementary Table 8) in both groups, showing that most LPS-induced ISG induction is primarily mediated by autocrine/paracrine signaling. The inhibitory effect of the IFNAR2 antagonist did not occur in the LPS-induced expression of "non-ISG" genes (Fig. 4D; Supplementary Table 8).

No defect in "in vitro" IFN activity in culture supernatant of LPS-stimulated cirrhotic PBMCs

Reduced IFN signaling activity due to decreased IFN secretion and/or an "*in vitro*" IFN-neutralizing activity in the supernatant of LPS-stimulated cirrhotic PBMCs could explain the defect in ISG induction in these cells. To address these questions, HuH7 cells were chosen because we found that they did not respond to LPS stimulation (data not shown); they can respond to IFNs but cannot produce IFNs themselves [29]. HuH7 cells were cul-


A
B
C
D
Relative mRNA expression (log2 scale)
TRIM22
IFIT1
OAS2
CXCL10
DDX58
IFIH1
MX2
GBP4
IFN score
TNF
CXCL8
IL1B
IL6
IL10
US
LPS
LPS + BRF
LPS + IgG2 mAb
LPS + anti-IFNAR2 mAb

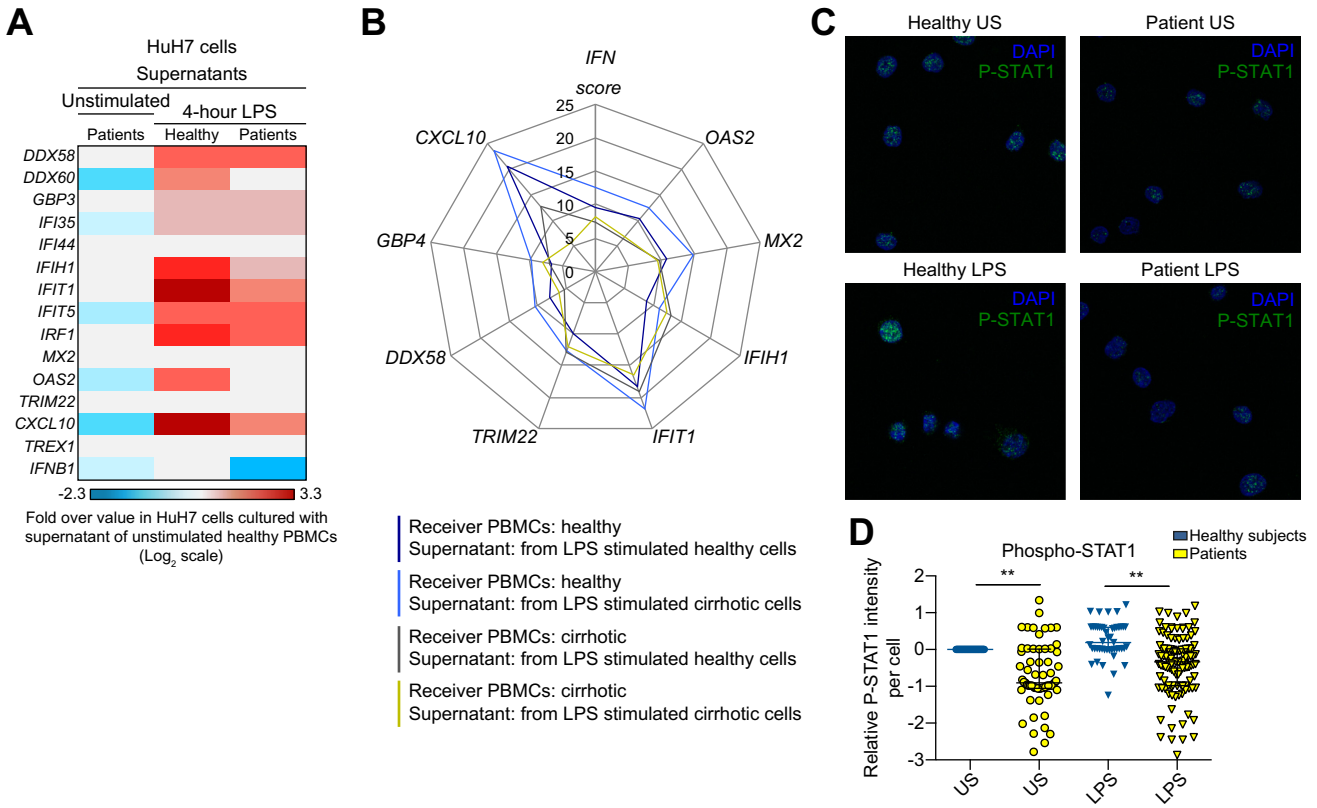


Fig. 5. The defect in LPS-elicited ISG induction is not due to decreased IFN production but to decreased IFN signaling with cirrhotic PBMCs. (A) ISGs (assessment, RT-qPCR) in HuH7 cells exposed to the supernatants of unstimulated and LPS-stimulated PBMCs from patients with cirrhosis and healthy subjects (n = 6 in each group). (B) Quantitative spider plot representation of changes in IFN score and its eight components in cross-stimulation experiments. 'Receiver' healthy PBMCs were with supernatant of LPS-stimulated healthy (dark blue) or cirrhotic (light blue) PBMCs; 'receiver' cirrhotic PBMCs were stimulated with supernatant of LPS-stimulated healthy (grey) or cirrhotic (yellow) PBMCs (n = 2 in each group). (C) Immunohistochemistry of nuclear phosphorylated STAT1 (P-STAT1) in cirrhotic and healthy PBMCs. (D) Levels of nuclear phospho-STAT1 in PBMCs from patients and healthy subjects (n = 3 and n = 2, respectively). US denotes unstimulated conditions. $^{**}p$ <0.01 (Mann-Whitney test). (This figure appears in colour on the web.)

tured with the supernatants of unstimulated and LPS-stimulated PBMCs from six patients with cirrhosis and six healthy subjects and ISG induction was measured using RT-qPCR. HuH7 cells exposed to supernatants of LPS-stimulated PBMCs exhibited an induction of 53% (8/15) of tested ISGs with "cirrhotic" supernatants and 66% (10/15) of tested ISGs with "healthy" supernatants (Fig. 5A), respectively. In addition, we performed cross-stimulation experiments using previously collected supernatants from 4 h LPS-stimulated cirrhotic and healthy cells to culture "*in vitro*" freshly isolated "receiver" PBMCs from two other patients and two other healthy subjects. Therefore, using four supernatants from patients and healthy subjects to stimulate "receiver" PBMCs, we found that ISG induction (reflected by an increase in the IFN score) caused by the "cirrhotic" supernatant was similar to that elicited by "healthy" supernatants. ISG induction was not restored to "receiver" cirrhotic PBMCs, when these cells were exposed to "healthy" supernatants (Fig. 5B). These findings do not confirm a defect in "*in vitro*" IFN activity in the supernatant of LPS-stimulated cirrhotic cells and suggest that the defect in ISG induction probably occurs as a result of intrinsic regulatory mechanisms.

Defect in STAT1 phosphorylation in LPS-stimulated cirrhotic PBMCs

IFNAR engagement induces phosphorylation of STAT1 and STAT2, which then are involved in induction of specific target ISGs [5]. We performed immunostaining and found that, in LPS-stimulated PBMCs, the levels of nuclear phosphorylated STAT1 (phospho-STAT1) were significantly lower in cirrhotic cells than in healthy cells (Fig. 5C and D). Since phospho-STAT1 is a potent activator of the formation of the ISGF3 complex [5], our results suggest that a defect in phospho-STAT1 may result in decreased canonical ISGF3 formation. In support of this we found that ISGs that are known to be "canonical" [5], including *OAS2*, *MX2*, *GBP4* had defective induction in LPS-stimulated cirrhotic PBMCs. However, there were also ISGs from this category (e.g., *EIF2AK2*, *IFI44*)

Fig. 4. LPS induces ISGs via an autocrine/paracrine signaling. (A) Effect of inhibition of cytokine secretion by brefeldin A (BRF) on LPS-induced ISGs in cirrhotic PBMCs (n = 5). (B) Effect of inhibition of cytokine secretion by brefeldin A on LPS-induced "non-ISG" genes in cirrhotic PBMCs (n = 5). (C) Effect of an IFNAR2 antagonist (IFNAR2 mAb, 30 μg/ml) and control isotype (IgG2 mAb, 30 μg/ml) on LPS-induced ISGs and IFN score in cirrhotic and healthy PBMCs (n = 5 and n = 6, respectively). (D) Effect of an IFNAR2 antagonist and control isotype on LPS-elicited "non-ISG" genes in cirrhotic and healthy PBMCs (n = 5 and n = 6, respectively). Values are median (IQR). US denotes unstimulated conditions. $^{*}p$ <0.05, $^{**}p$ <0.01 (Wilcoxon signed-rank test). (This figure appears in colour on the web.)

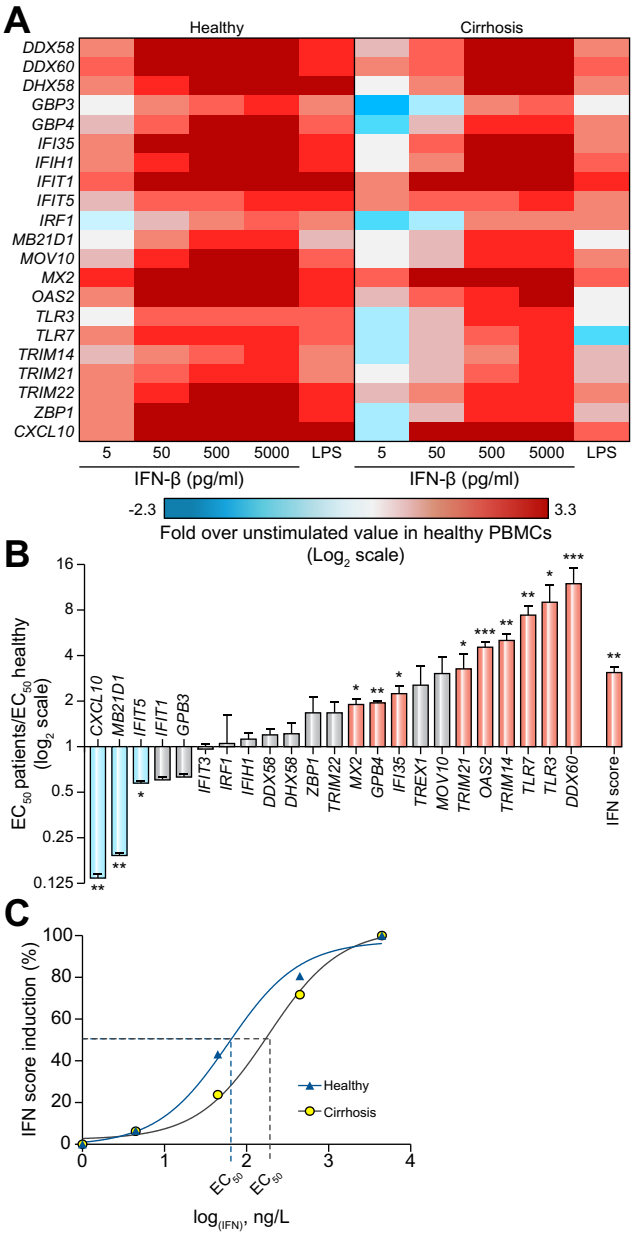


Fig. 6. Cirrhotic PBMCs have defective ISG response to IFN-β. (A) 23 ISGs in cirrhotic and healthy PBMCs stimulated with increasing IFN-β concentrations (n = 10 and n = 8, respectively). (B) ISG sensitivities to IFN-β: cirrhotic *vs.* healthy PBMCs. EC_{50} (IFN concentration that half-maximally induced ISG) was determined and $EC_{50\ patient}$/ $EC_{50\ healthy}$ ratio was calculated. (C) IFN score changes with IFN-β. US, unstimulated. $^{*}p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.0001 (unpaired *t* test). (This figure appears in colour on the web.)

that were "normally" induced in LPS-stimulated cirrhotic PBMCs, suggesting that, as shown elsewhere [5], certain mechanisms can compensate for the defect in the active form of STAT1 in a gene-specific manner. Interestingly, in LPS-stimulated cirrhotic PBMCs, certain ISGs that are known targets for non-canonical IFN signaling pathways [5] had defective induction (*CXCL10*, *TLR3*) while others had "normal" induction (*MCOLN2*, *AIM2*). Together these findings suggest that the defect in LPS-induced ISGs is a gene-specific but not signal-specific phenomenon.

Defective ISG induction in IFN-β-stimulated cirrhotic PBMCs

We evaluated ISG sensitivity to IFN-β across a 10^3 range of concentrations in cirrhotic and healthy PBMCs. Twenty-three ISGs with weak LPS induction in cirrhotic PBMCs were investigated. There was a concentration-dependent increase in the induction of all ISGs whatever the origin of the cells (Fig. 6A), but, as expected [5,30], sensitivity to IFN-β was different among ISGs, with a large difference in the EC_{50} for half-max response (Supplementary Table 9). When ISG sensitivities to IFN-β were compared between cirrhotic and healthy cells, three different classes of ISGs were identified (Fig. 6B): a class of 10/23 (43.5%) genes with less sensitivity (i.e., higher EC_{50}) in cirrhotic cells; a class of 3/23 (13.0%) genes with greater sensitivity (i.e., lower EC_{50}) in these cells; and a class of 10/23 (43.5%) genes with similar sensitivities to IFN-β in both groups. Of note, the EC_{50} value of the IFN score was significantly higher in cirrhotic cells than in healthy cells (Fig. 6C), essentially measuring the decreased sensitivity of four of its components. Together, these findings suggest that cirrhotic PBMCs have intrinsically altered ISG sensitivity to IFN-β.

Constitutive ISG under-expression correlates with defective LPS-elicited ISG induction

Constitutive ISG expression may influence the level of induction in response to type I IFNs [5,22]. Using our set of 48 ISGs, we analyzed, for each gene, the correlation between baseline expression and expression at 4 h following LPS, in cirrhotic and healthy PBMCs. In both groups (Supplementary Table S10), for most ISG pairs (36/48 in patients [representative ISGs in Supplementary Fig. 3]; 30/48 in healthy subjects), we found a significant positive correlation between baseline and LPS-induced expressions, consistent with the view that LPS-induced ISG levels depend, at least in part, on their baseline expression levels. Moreover, in both groups, baseline IFN score values significantly correlated with LPS-induced values (Supplementary Fig. 3). Of note, in cirrhotic PBMCs, there were large inter-individual variations in baseline ISG expression which ranged from "normal values" to very low values (Fig. 2A and B; Supplementary Table 5C). Nevertheless, the median baseline values for expression of 25/48 (52%) ISGs (Fig. 2B; Supplementary Table 5C) and the IFN score (Fig. 3A) were significantly lower in cirrhotic than healthy PBMCs. Therefore, constitutive ISG under-expression contributed to the defective LPS-evoked ISG induction in cirrhotic PBMCs.

ISGS are "tolerizable"

Repeated LPS challenge induces tolerance to LPS, which aims to limit inflammation. It is not known if ISGs are "tolerizable" or not, particularly in cirrhosis. We first stimulated PBMCs from patients and healthy subjects with 100 ng/ml LPS for 24 h. The culture medium was then changed and cells were left unstimulated or stimulated for 4 h with a low LPS concentration (10 ng/ml) (Fig. 7A). Certain other cells were left unstimulated throughout the 28 h, resulting in four types of cells: naïve cells, naïve cells that were stimulated only once with 10 ng/ml LPS, tolerant cells (stimulated once with 100 ng/ml LPS) and tolerant cells that were exposed to two successive LPS stimulations (Fig. 7A). All tested ISGs were "tolerizable" both in cirrhotic and healthy immune cells, i.e., their expression was significantly lower after two LPS stimulations than after one (Fig. 7B). Unexpectedly in

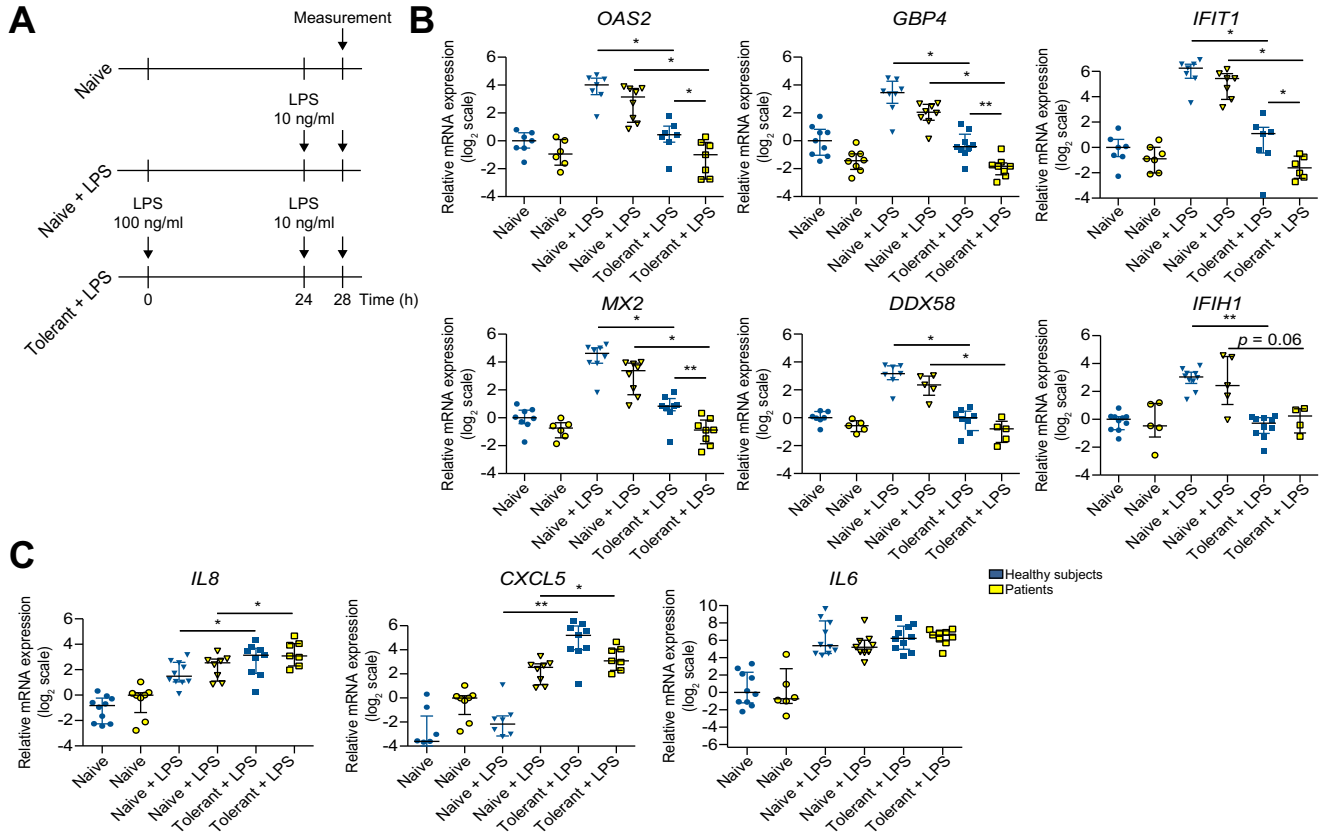


Fig. 7. ISGs are "tolerizable". (A) Cirrhotic and healthy PBMCs (n = 9 and n = 10, respectively) were left unstimulated (naïve, N) or stimulated with 100 ng/ml LPS for 24 h (tolerant, T), washed with PBS and given media (N) or 10 ng/ml LPS (N + L, T + L). Only, three experimental groups (N, N + L, T + L) are shown here. (B) ISGs and "non-ISGs" in cirrhotic and healthy PBMCs in the three displayed groups (N, N + L, T + L). $^{*}p$ <0.05, $^{**}p$ <0.01 (Wilcoxon signed-rank test or Mann-Whitney test). (This figure appears in colour on the web.)

both cirrhotic and healthy cells, pro-inflammatory cytokine genes such as *CXCL8*, *IL6*, *CXCL1* and *CXCL5* had similar or even higher expression after two LPS stimulations than one, defining these genes as "non-tolerizeable" (Fig. 7C; Supplementary Table 11). These results show that ISG induction in immune cells exposed to repeated LPS stimulation is tightly and specifically inhibited through tolerance mechanisms.

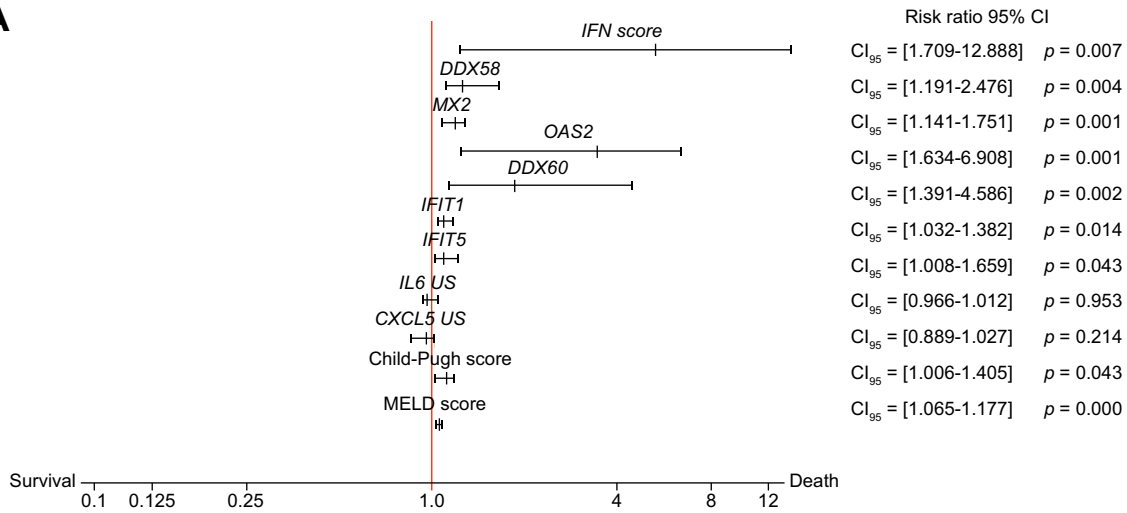
Baseline ISG expression is related to outcome in cirrhosis

We prospectively followed-up a cohort of patients after measurement of PBMC ISG expression at enrollment. Forty-one patients died during a median follow-up of 6.55 (1.32–12.60) months. A univariate Cox model showed that higher mortality was significantly associated with a higher MELD score, a higher IFN score in unstimulated cells, and higher expressions of *DDX58*, *MX2*, *OAS2*, *DDX60*, *IFI44*, *IFIT1*, *IFIT5* and *IRF1* in unstimulated cells (Fig. 8A; Supplementary Table 12). Unlike ISGs, cytokine gene expression (including the ISG *CXCL10*) did not significantly predict death in unstimulated cells (Fig. 8A; Supplementary Table 12). Only one (*IFI35*) of the genes measured in LPS-stimulated cells significantly predicted death (Supplementary Table 12). Bivariate analysis including the baseline IFN and MELD scores only showed the former to be significantly predictive of death (Fig. 8B). These results are mainly explained by the intrinsic prognostic value of higher baseline *MX2*, *OAS2*, and *DDX58*. Finally, higher baseline expressions of *IFI44* and *DDX60* were significant predictors of death, independent of the MELD score (Fig. 8B).

Discussion

This *ex vivo* study found an ISG induction in LPS-stimulated PBMCs from patients with decompensated alcoholic cirrhosis and healthy subjects. These results confirm previous findings [6–10] showing that LPS, a Gram-negative bacteria component, can induce an innate immune response including the induction of "antiviral" ISGs, in the absence of any viral pathogens. The present study found the following results related to LPS-induced ISGs: 1) Cirrhotic PBMCs (and to a lesser extent cirrhotic monocytes) exhibited a defect in LPS-induced, IFN-mediated, autocrine/paracrine induction of a significant number of ISGs compared to healthy PBMCs or monocytes. 2) The decrease in LPS-inducible ISGs expression was not due to overall "immune paralysis" of cirrhotic PBMCs. Thus, LPS-stimulated cirrhotic PBMCs could express large amounts of inflammatory cytokines and chemokines, some of these with stronger induction in cirrhotic than in healthy PBMCs. 3) Defective ISG induction in LPS-stimulated cirrhotic PBMCs was not caused by decreased IFN secretion or neutralization of secreted IFN in the extracellular milieu but by intracellular alterations that are gene-specific

A



B

	Number of events	Total number of patients	Risk ratio	95% CI for risk ratio		*p* value
				Lower	Upper	
First model	14	43				
IFN score basal			3.583	1.177	10.905	0.025
MELD score			1.071	0.993	1.155	0.074
Second model	21	56				
MX2 basal expression			1.346	1.077	1.681	0.009
MELD score			1.091	1.020	1.167	0.011
Third model	20	55				
OAS2 basal expression			2.492	1.053	5.901	0.038
MELD score			1.060	0.984	1.141	0.122
Fourth model	10	26				
IFI35 basal expression			1.393	1.043	1.861	0.025
MELD score			1.178	1.026	1.351	0.020
Fifth model	9	17				
IFI44 basal expression			1.635	1.017	2.627	0.042
MELD score			1.032	0.936	1.136	0.529

Fig. 8. Baseline ISG expression is related to patients' outcome. (A) Results of analysis using a univariate Cox model. (B) Results of analysis using a bivariate Cox model. (This figure appears in colour on the web.)

rather than signal-specific. 4) Defective ISG induction (which was captured by an 8-ISG signature [IFN score]) is a hallmark of cirrhotic PBMCs because it was found not only with LPS but also with two other PAMPs (poly (I:C); poly (I:C)-plus-lipofectamine) or IFN-β stimulation. However, the extent of the defect in ISG induction was contextual, depending on the nature of the stimulus (the number of defective ISGs being larger with LPS than with other PAMPs or IFN-β). 5) We found that LPS-elicited ISG induction was a highly "tolerizable" process in both cirrhotic and healthy PBMCs, suggesting that sustained activation of the IFN conduit would otherwise be detrimental to the organism. This hypothesis is supported by results in mouse models of endotoxin shock, auto-immune diseases and Mendelian interferonopathies, showing that IFN signaling is a major contributor to tissue damage and poor outcome (See Supplemental material, Supplementary Table 1) [15].

In this study, the defect in LPS-elicited ISG induction was observed in PBMCs from patients with alcoholic cirrhosis but not in those with HCV-related cirrhosis (the severity of cirrhosis was similar in both groups). These results suggest that defective ISG induction is related to the alcoholic etiology of cirrhosis rather than the severity of liver disease. Thus, patients with alcoholic cirrhosis did not exhibit a significant correlation between the LPS-induced IFN score and the MELD score. Accordingly, alcohol consumption may play a role in the existence of defective ISG induction via a direct effect of alcohol on the immune response to LPS or by promoting LPS translocation [31] and subsequent induction of tolerance mechanisms.

In this study, at baseline, the IFN score and the level of constitutive expression of several ISGs was significantly lower in cirrhotic PBMCs than in healthy PBMCs. Interestingly, the lower baseline ISG expression or IFN score value, the lower LPS-elicited gene induction or IFN score value, suggesting that constitutive ISG under-expression contributes to defective LPS-elicited induction.

One important finding in this study was that baseline IFN score, or the expression level of score components (*OAS2*, *MX2*, *DDX58*) as well as two other ISGs (*IFI44*, *DDX60*), were significant

predictors of death, independently of the MELD score. The higher the score or ISG expression, the higher the risk of death. Of note, in patients, baseline expression levels of ISGs of prognostic importance and baseline values of the IFN score ranged from "normal" values to very low values. Therefore, our results suggest that even "normal" baseline values for these ISGs or IFN score are "too high" for patients as indicated by their significant correlation with mortality. At the opposite, the strong ISG under-expression measured in some of our patients might be a result of mechanisms reminiscent to tolerance.

Patients with acutely decompensated cirrhosis develop systemic inflammation in response to different triggers [32]. In these patients, the higher the systemic inflammatory response, the higher the risk of developing organ failure(s) and death [32]. Therefore, mechanisms underlying systemic inflammation are important in determining the prognosis of cirrhosis. In non-cirrhotic immune cells, sustained constitutive expression of key transducers of the STAT family (which are ISGs) have an effect on immunological activity because it increases the immune cell sensitivity to acutely produced IFN or other STAT-dependent cytokines (e.g., IL-6, CSF-1). Therefore, among patients with cirrhosis, those with the highest constitutive ISG expression (including that of key STAT transducers) could have, in the context of acute decompensation, an enhanced circulating immune cell sensitivity to acutely produced IFN or other cytokines, resulting in further IFN production, systemic inflammation and subsequent increased risk of tissue damage and death (Supplementary Fig. 4).

In conclusion, PBMCs from patients with decompensated alcoholic cirrhosis exhibit an *ex vivo* defect in constitutive and LPS-induced ISG expression. Constitutive ISG under-expression seems to be an important contributor to defective LPS-elicited induction of these genes. Our finding that higher baseline PBMC ISG expression was associated with higher risk of death, is consistent with a contribution of constitutive ISG expression in systemic immune cells to the prognosis of alcoholic cirrhosis.

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Conflict of interest

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

Authors' contributions

Study concept and design (RM, EW); acquisition of data (EW, PER, MF, MG, MDC, CM, TG, MHN); analysis and interpretation of data (EW, PER, MF, MG, MDC, AC, Ra. M, JHW, MHN, PDLG, RCM, RM); drafting of the manuscript (EW, AC, RCM, RM); critical revision of the manuscript for important intellectual content (TG, DL, DV, FD, RCM, CPB, SL); statistical analysis (EW, PER, RaM, RM); study supervision (RM).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2016.12.008.

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Author names in bold designate shared co-authorship

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