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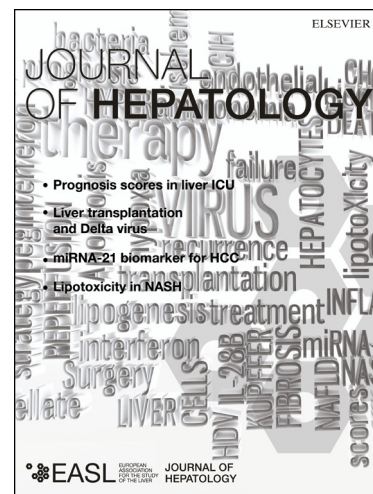
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IFN- γ inhibits liver progenitor cell proliferation in HBV-infected patients and in 3,5-diethoxycarbonyl-1,4-dihydrocollidine diet-fed mice

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Abbreviations

ALT: alanine aminotransferase; α -SMA: α -smooth muscle actin; BMOL: bipotential murine oval liver; BrdU: Bromodeoxyuridine; CCl₄: carbon tetrachloride; CDE: choline deficient, ethionine supplemented diet; CK7: cytokeratin-7; CK19: cytokeratin-19; DDC: 3,5-diethoxycarbonyl-1,4-dihydrocollidine; DR, ductular reaction; ECM: extracellular matrix; HSC: hepatic stellate cell; IHC: immunohistochemistry; IHLC: intermediate hepatocyte-like cell; IRF-1: interferon regulator factor-1; LPC: liver progenitor cells; LPS, lipopolysaccharide; MPK: muscle pyruvate kinase; NK cell, natural killer cell; NKT cell, natural killer T cell; PCNA: proliferating cell nuclear antigen; STAT1: signal transducer and activator of transcription 1; Th1, T helper 1.

Abstract

Background & Aims: Proliferation of liver progenitor cells (LPCs) is associated with inflammation and fibrosis in chronic liver diseases. However, how inflammation and fibrosis affect LPCs remains obscure. **Methods:** We examined the role of interferon (IFN)- γ , an important pro-inflammatory and anti-fibrotic cytokine, in LPC expansion in HBV-infected patients and in mice challenged with 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC)- or choline-deficient, ethionine-supplemented (CDE) diet as well as in primary LPCs and LPC cell line. **Results:** The CK19 staining scores correlated with inflammation and fibrosis grades in the livers from 110 HBV-infected patients. Nine-month IFN- γ treatment decreased LPC numbers, inflammation, and fibrosis in these HBV-infected patients. Similarly, a two-week IFN- γ treatment also decreased LPC activation in DDC-treated mice. Disruption of IFN- γ or its signaling components (eg. IFNGR, STAT1, and IRF-1) increased LPC proliferation and liver fibrosis in DDC-fed mice. In contrast, deletion of IFN- γ did not increase but rather slightly reduced LPC proliferation in CDE-fed mice. *In vitro*, IFN- γ attenuated proliferation of the LPC cell line BMOL cells and of primary LPCs from wild-type mice, but not STAT1^{-/-} or IRF-1^{-/-} mice. Furthermore, co-culture assays suggest that IFN- γ can indirectly promote LPC proliferation via the activation of macrophages but attenuate it via the inhibition of hepatic stellate cells. **Conclusion:** IFN- γ inhibits LPC expansion via the direct inhibition of LPC proliferation and indirect attenuation of liver fibrosis in the DDC model but it may also enhance LPC expansion via the promotion of inflammation in the CDE model; thereby playing dual roles in regulating LPC proliferation *in vivo*.

Keywords: cytokeratin-19, cytokeratin-7, STAT1, IRF-1, hepatic stellate cells, macrophages

Introduction

Liver progenitor cells (LPCs), which are denoted as oval cells in rodents, reside in the smallest and most peripheral branches of the biliary tree, the ductules, and canals of Hering [1-6]. LPCs are activated in chronic liver diseases and participate in liver regeneration, fibrogenesis, and hepatocarcinogenesis. Upon severe liver injury with hepatocyte and cholangiocyte necrosis/apoptosis, the bi-potential LPCs may differentiate into hepatocytes or cholangiocytes, depending on which cell type is the most damaged [1-6]. LPC activation occurs in regions of periportal fibrogenesis in patients with chronic liver disease, as well as in a variety of rodent models [1-6]. Currently, the mechanisms underlying LPC activation and proliferation are not fully understood. Many growth factors and cytokines that promote hepatocyte proliferation and survival have also been found to promote LPC proliferation [1-6], with the exception of interferon-gamma (IFN- γ) [7]. IFN- γ has been well documented to induce cell cycle arrest and apoptosis in many cell types, including hepatocytes and hepatic stellate cells (HSCs) [8-11]. Although IFN- γ and its downstream target genes are activated in oval cell reaction in rat liver regenerating after a 70% partial hepatectomy combined with the feeding of 2-acetylaminofluorene [12]; however, the effects of IFN- γ on LPC proliferation have been inconclusive [8, 13-15].

IFN- γ mediates its effects by binding to IFN- γ receptor 1 (IFNGR1) and IFNGR2, which induces Janus kinase phosphorylation and subsequently activates multiple signal transducers and activators of transcription (STATs), with STAT1 being predominantly activated in hepatocytes [9]. Activated STAT1 forms a dimer and translocates into the nucleus to induce the transcription of many genes, including IFN- γ regulatory factor 1 (IRF-1), p21, and caspase 3, that promote cell apoptosis and cell cycle arrest [9, 10]. In the liver, IFN- γ , which is mainly produced by natural killer (NK), NKT, and Th1 cells, may target all types of liver and immune cells. In this context, IFN- γ mediates a variety of important functions by regulating anti-viral, anti-tumor, pro-inflammatory, pro-apoptotic, anti-proliferative, and anti-fibrotic responses [9, 10]. For example, the pro-inflammatory effect of IFN- γ in the liver is mediated, at least in part, by the activation of Kupffer cells [16] and macrophages [17]. IFN- γ exerts an anti-fibrotic effect by directly inducing HSC apoptosis and cell cycle arrest

via an STAT1-dependent manner [11]. Hepatocytes express high levels of IFNGR1 and IFNGR2 and are responsive to IFN- γ stimulation. IFN- γ induces hepatocyte apoptosis and cell cycle arrest via STAT1- and IRF-dependent mechanisms [9, 10]. Although the anti-proliferative effect of IFN- γ has been well documented in HSCs and hepatocytes [8-11], the effect of IFN- γ on LPC proliferation remains unclear [8, 13-15].

In the present study, we examined the effects of IFN- γ on human LPC proliferation/ductular reaction (DR) in hepatitis B virus (HBV)-infected patients with tissue samples collected in a previous anti-fibrotic clinical trial [18]. The roles of IFN- γ , its downstream signaling molecules, and its target genes in mouse LPC proliferation were further examined in mice that were fed a 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) or choline-deficient, ethionine-supplemented (CDE) diet, as well as in cell culture models.

Materials and Methods

Human liver specimens

A total of 110 biopsied liver tissue specimens from patients with chronic HBV infection [19] and 18 patients with chronic HBV infection who underwent a 9-month IFN- γ treatment in a previous clinical trial [18] were investigated. The local ethics committees approved the study protocol involving the use of human samples, and every patient provided written informed consent.

Other materials are described in the Supplementary Materials.

Results

LPCs are activated in HBV patients and are suppressed following IFN- γ therapy

LPCs represent a dynamic cellular compartment that continuously changes its morphology and phenotype in relation to its differentiation state. This may be the reason why there are no specific LPC markers that can distinguish biliary cells, ductular cells, and LPCs [3]. In the current paper, we performed immunohistochemistry analyses by using CK19, which is a well-recognized LPC marker so far. However, CK19 is also a marker for cholangiocytes. Combined with morphology feature of LPCs, CK19 could successfully identify LPCs [20] and we defined CK19⁺ LPCs by counting the number of isolated CK19⁺ cells in the periportal area of the lobules. Because LPCs are not necessarily isolated and ductular reactions (DRs) may also arise from LPCs in addition from proliferation of pre-existing cholangiocytes and rarely from biliary metaplasia of hepatocytes [5, 20]. Therefore, we also counted CK19⁺ DRs in these human liver tissues.

Supplementary Figure 1 exemplifies the DRs, LPCs, and intermediate hepatocyte-like cells (IHLCS) in the livers of HBV patients. To correlate LPC activation/DR and severity of disease in liver tissue, CK19 (marker of LPC/DR) [21] and CK-7 (marker of IHLCS) [21] were scored by immunohistochemistry analyses of 110 liver tissue specimens from HBV patients. Both DR grades and numbers of activated LPCs correlated positively with inflammatory grades and fibrotic stages (Supplementary Fig. 1). IHLCS were found to correlate positively with fibrotic stages ($r = 0.17$, $p < 0.05$) (data not shown).

The above data suggest that inflammation promotes LPC activation/DRs in HBV patients. However, the underlying mechanisms of LPC activation/DRs remain largely unknown. Several previous studies suggest that inflammatory cells may promote LPC proliferation in mice via the production of IFN- γ [14]. To explore the relationship between IFN- γ and LPC expansion, we first examined IFN- γ expression by immunohistochemical analyses of 30 HBV-infected liver tissue specimens. Unexpectedly, IFN- γ expression was detected at very low levels in these HBV-

infected livers (Fig. 1A), suggesting that endogenous IFN- γ is not involved in LPC activation/DR in these liver tissues.

Next we examined whether IFN- γ therapy regulated LPC activation/DRs in HBV-infected patients who were treated with IFN- γ for 9 months in a published anti-fibrotic clinical trial [18]. The results from this clinical trial revealed that IFN- γ treatment did not reduce viral load but ameliorated liver inflammation and fibrosis in these HBV patients [18]. In the present study, we examined the number of LPCs/DRs in biopsy samples from 18 HBV patients before and following a 9-month IFN- γ treatment. This treatment did not significantly promote HBV seroconversion [18], but it markedly reduced the number of CK19⁺ LPCs and DRs in 13 out of 18 HBV patients (Fig. 1B-D and supplementary Table 1). The number of LPCs was increased in one patient, while the number of DRs was increased in two patients after therapy (Fig. 1B-D and supplementary Table 1). The data imply that LPC numbers are decreased with a concomitant reduction in inflammatory and fibrotic responses in most HBV patients after 9 months of IFN- γ therapy.

IFN- γ treatment inhibits liver inflammation, fibrosis, and LPC expansion in DDC-fed mice

To further examine the effect of IFN- γ treatment on LPC activation, a mouse model with LPC activation induced by DDC diet feeding was used. As illustrated in Figs. 1E-F, treatment with IFN- γ for 2 weeks markedly inhibited liver fibrosis in this animal model, as demonstrated by Sirius red and α -SMA staining. Interestingly, IFN- γ treatment also markedly reduced liver inflammation in DDC-fed mice. Moreover, immunohistochemistry with CK19 revealed that the number of CK19⁺ cells was lower in the IFN- γ -treated DDC group compared with the DDC control group (Figs. 1E and 1G). PCNA (a cell proliferation marker) staining analysis showed that the proliferation of nonparenchymal cells was upregulated after DDC feeding. PCNA⁺ cells were less prevalent in IFN- γ -treated mice (Figs. 1E and 1H).

Disruption of the IFN- γ downstream signaling pathways enhances liver inflammation, fibrosis, and LPC expansion in DDC-fed mice

We previously demonstrated that IFN- γ ^{-/-} mice are more susceptible to DDC-induced liver fibrosis, suggesting that endogenous IFN- γ inhibits liver fibrosis in the DDC model [22]. To examine the role of endogenous IFN- γ in liver inflammation and fibrosis, several strains of knockout mice with genetic deletions in the IFN- γ downstream signaling pathways were used. As illustrated in Figs. 2A-B, IFNGR1^{-/-}, STAT1^{-/-}, and IRF-1^{-/-} mice had a greater degree of liver fibrosis than wild-type mice. Moreover, H&E staining showed that, compared with wild-type mice, these three strains of knockout mice exhibited significantly more liver inflammation after DDC feeding (Fig. 2C).

We also examined DDC-induced LPC expansion in wild-type and knockout mice. Compared with wild-type mice, the numbers of LPCs were higher in all genetically modified mice after 2- or 4-week DDC feeding periods (Figs. 2D). Among these genetically modified mice, IFN- γ ^{-/-} mice exhibited the highest LPC number, which was approximately three-fold higher than in wild-type mice. The numbers of LPCs in IFNGR1^{-/-}, STAT1^{-/-}, and IRF-1^{-/-} mice were approximately two-fold greater than in wild-type mice (Fig. 2E).

The above data suggest that IFN- γ treatment inhibits LPC proliferation *in vivo*. To examine whether STAT1 and IRF-1 mediate the IFN- γ inhibition of LPC proliferation *in vivo*, BrdU and CK19 double staining was performed to compare LPC proliferation in DDC-fed STAT1^{-/-}, IRF-1^{-/-}, and wild-type mice. As illustrated in Fig. 3A, STAT1^{-/-} and IRF-1^{-/-} mice had a significantly higher number of BrdU and pan-CK double-positive LPCs compared with wild-type mice. These results indicate that the proliferation of LPCs is significantly increased in mice with STAT1 or IRF-1 deficiency.

IFN- γ inhibits LPC proliferation *in vitro* via STAT1- and IRF-1-dependent mechanisms

To investigate the direct impact of IFN- γ on LPC proliferation, we examined the effect of IFN- γ on the proliferation of BMOL cells, a murine LPC cell line. As shown in Fig.

3B, incubation with IFN- γ for 24 hours inhibited the proliferation of BMOL cells. Consistent with the BrdU incorporation data, IFN- γ incubation also decreased PCNA expression in these cells with strong activation of pSTAT1 (Fig. 3C). Moreover, *in vitro* treatment with IFN- γ also inhibited BrdU incorporation in primary LPCs from wild-type mice but not from STAT1^{-/-} or IRF-1^{-/-} mice (Fig. 3D).

IFN- γ indirectly regulates LPC proliferation by targeting macrophages or HSCs

The above data suggest that IFN- γ inhibits LPC proliferation in HBV-infected patients and DDC-fed mice. However, previous reports showed that IFN- γ ^{-/-} mice had a similar number of A6⁺ LPCs but decreased muscle pyruvate kinase (MPK)⁺ LPC expansion compared with wild-type mice after being fed a CDE diet [13]. We also examined LPC expansion in the CDE model and quantified LPCs by pan-CK staining. As illustrated in Fig. 4A, the number of LPCs was slightly but not significantly reduced in IFN- γ ^{-/-} mice compared with wild-type mice after CDE feeding.

In the CDE model, inflammatory cells, especially invading macrophages, played critical roles in supporting LPC growth by producing cytokines such as TNF- α , IL-6, and TWEAK [23-25]. In contrast, in the DDC model, LPCs are surrounded by activated HSCs, which produce large amounts of collagen that may preclude cellular contact between macrophages and LPCs [26]. Given these considerations, HSCs, but not macrophages, may affect LPC expansion in the DDC model. Because IFN- γ is a potent activator of macrophages, we hypothesized that in the CDE model, IFN- γ may activate macrophages to produce cytokines that promote LPC proliferation. To test this hypothesis, macrophages were treated with IFN- γ *in vitro*, and cytokines were then measured. As shown in Fig. 4B, IFN- γ treatment upregulated the expression of TNF- α and IL-6, but not TWEAK, in macrophages.

We then compared the hepatic expression of IL-6 and TNF- α in IFN- γ ^{-/-} and wild-type mice after being fed a DDC or CDE diet. As illustrated in Figs. 4C-D, the expression of IL-6 and TNF- α was upregulated in wild-type mice undergoing a DDC diet or CDE diet. After DDC feeding, the upregulation was comparable in IFN- γ ^{-/-} and wild-type mice. Following CDE feeding, the IL-6 expression was significantly lower in IFN- γ ^{-/-}

mice than in wild-type mice. This result suggests that IFN- γ plays a role in promoting IL-6 production in the CDE but not in the DDC model.

To mimic the interaction between inflammatory cells and LPCs in the CDE model, LPCs isolated from IFNGR1^{-/-} mice (to exclude the direct effects of IFN- γ on LPCs) were co-cultured with intrahepatic lymphocytes (IHLs), Kupffer cells and peritoneal macrophages from wild-type mice in the presence or absence of IFN- γ . LPC proliferation was then quantified by pan-CK and BrdU double staining. As shown in Fig. 4E, IHLs or IFN- γ -treated IHLs did not alter proliferation of IFNGR1^{-/-} LPCs. However, IFN- γ -treated macrophages augmented proliferation of IFNGR1^{-/-} LPCs. In addition, Kupffer cells with or without IFN- γ treatment did not influence IFNGR1^{-/-} LPC growth. These data further supported that invading macrophage activation promoted LPC growth in CDE model.

Similarly, HSCs and IFNGR1^{-/-} LPCs were also co-cultured in the presence or absence of IFN- γ . As illustrated in Fig. 4E, the co-culture with HSCs enhanced IFNGR1^{-/-} LPC proliferation. However, such enhancement was not observed in the group of LPCs co-cultured with IFN- γ -treated HSCs.

Discussion

Although the anti-proliferative effects of IFN- γ on hepatocytes and HSCs have been well documented [8-11], the data on the effects of IFN- γ on LPC proliferation have been controversial [8, 13-15]. In this study, we provided several lines of evidence suggesting that IFN- γ plays an important role in inhibiting LPC expansion in DDC-fed mice and cultured LPCs *in vitro* involving STAT1- and IRF-1-dependent pathways. First, IFN- γ treatment attenuated the proliferation of BMOL cells and primary mouse LPCs *in vitro* with predominant STAT1 activation. Such inhibitory effects were not observed in primary STAT1^{-/-} or IRF-1^{-/-} LPCs. Second, IFN- γ ^{-/-} mice had a much greater number of pan-CK⁺LPCs than wild-type mice after being fed a DDC diet. Third, compared with wild-type mice, several strains of genetically modified mice with IFNGR1, STAT1, or IRF-1 deficiency displayed remarkably increased LPC proliferation after DDC challenge. In addition, we have demonstrated that IFN- γ may indirectly suppress LPC proliferation by attenuating HSC activation or indirectly augmenting LPC proliferation by activating macrophages. We have integrated these findings into a model (Fig. 4F) that depicts the complex roles of IFN- γ plays in controlling LPC proliferation.

IFN- γ directly inhibits LPC proliferation: IFN- γ has been well documented to inhibit the proliferation of many cell types, including hepatocytes and HSCs, via the activation of STAT1 [8-11]. Treatment with IFN- γ markedly inhibited the proliferation of BMOL cells and primary mouse LPCs *in vitro*. This inhibitory effect is likely mediated via the activation of STAT1, a key transcription factor that inhibits cell proliferation. Indeed, IFN- γ treatment induced strong STAT1 activation, and the inhibitory effect of IFN- γ was diminished in STAT1^{-/-}LPCs. Our findings are consistent with those of a previous study by Isfort et al.[15] showing that IFN- γ inhibited the proliferation of several LPC cell lines *in vitro*. In contrast, other study [8] have shown that IFN- γ treatment had a small stimulatory effect on DNA replication in the LPC cell line LE6 cells based on a [H^3] thymidine incorporation assay. IFN- γ itself had no effect on the proliferation of the LPC cell line LE2, but the combination of IFN- γ with TNF- α or LPS promoted LE2 cell proliferation [8]. The discrepancies between these studies [8] and our study as well as the study by Isfort et al [15] may be due to the different LPC cell lines, culture

and treatment conditions, and assays used to quantify cell proliferation. In addition, treatment with type I IFN- α , a cytokine that also predominately activates STAT1, significantly suppressed the LPC cell line PIL cell proliferation *in vitro* [27]. Taken together, our model is in accord with IFN- γ directly inhibiting LPC proliferation *via* STAT1 activation.

IFN- γ inhibits LPC proliferation and fibrosis, thereby inhibiting LPC expansion in the DDC model: In addition to direct inhibition of LPC proliferation, IFN- γ may also indirectly affect LPC proliferation by targeting other types of liver cells, such as HSCs. This may occur given that IFNGR1 and IFNGR2 are ubiquitously expressed. In the DDC model, activated LPCs are localized in portal areas and surrounded by a thick layer formed through activated HSCs and matrix proteins. This likely affects macrophage functions and abilities to communicate with LPCs. Such interpretation anticipates that HSCs play a more important role in promoting LPC proliferation compared to macrophages in this model [26]. Indeed, our data show that co-culture with HSCs markedly increased LPC proliferation (Fig. 4). IFN- γ -treated HSCs had a significantly reduced ability to stimulate the proliferation of IFNGR^{-/-} LPCs (IFNGR^{-/-} LPCs were used to exclude the direct effect of IFN- γ on LPCs). Moreover, compared with DDC-fed wild-type mice, liver fibrosis was markedly enhanced in several strains of IFN- γ ^{-/-}, IFNGR1^{-/-}, STAT1^{-/-}, and IRF-1^{-/-} mice after DDC diet feeding (Figs. 2A-C), which positively correlated with the increased LPC proliferation in these mice (Figs. 2D-E). In addition, IFN- γ attenuated both fibrosis and LPC activation in the DDC-fed mice (Fig. 1E-H). Collectively, in the DDC model, HSCs likely promote LPC proliferation via the production of a large array of growth factors [28]. IFN- γ inhibits HSC activation and liver fibrosis and subsequently blocks the stimulatory effect of HSCs on LPC proliferation.

IFN- γ promotes liver inflammation and injury, thereby potentiating LPC expansion in the CDE model: After being fed a CDE diet, IFN- γ ^{-/-} mice exhibited slightly decreased rather than increased pan-CK LPC activation (Fig. 4A). A previous study reported that after CDE feeding, IFN- γ ^{-/-} mice had a similar number of A6⁺ LPCs but reduced MPK⁺ LPC expansion compared with WT mice [13]. It is believed that MPK stains LPCs (oval cells) in the periportal and parenchymal areas, as well as small

hepatocytes [29]; whereas A6 and pan-CK mainly detect ductal and periductal LPCs with a 99% overlap in staining [30]. This suggests that IFN- γ does not have a role in controlling A6⁺ or CK19⁺ LPC proliferation, but may have a role in promoting LPC-mediated generation of hepatocytes in the CDE model [13].

IFN- γ inhibits LPC proliferation *in vitro*. How may this result be reconciled with the findings that wild-type and IFN- γ ^{-/-} mice exhibited a similar number of A6⁺ or CK19⁺ LPCs after being fed a CDE diet (reference [13] and this study)? In the CDE model, activated LPCs are interspersed and predominantly associated with infiltrating macrophages, suggesting that macrophages, rather than HSCs, play a key role in promoting LPC proliferation [23, 26]. Because IFN- γ is a key pro-inflammatory cytokine that promotes liver inflammation and injury [9, 10], it may promote LPC proliferation *in vivo* by indirectly stimulating the production of several LPC growth mediators in the CDE model. This notion is supported by several lines of evidence. First, co-culture with macrophages had no effect on LPC proliferation *in vitro*, but IFN- γ -treated macrophages markedly enhanced LPC proliferation (Fig. 4E). Second, IFN- γ treatment markedly stimulated macrophages to produce IL-6 and TNF- α (Fig. 4B), which stimulate LPC growth [7]. Third, the hepatic expression of IL-6 was lower in CDE-fed IFN- γ ^{-/-} mice compared with CDE-fed wild-type mice, but was comparable in DDC-fed IFN- γ ^{-/-} and wild-type mice (Fig. 4D). This suggests that IFN- γ plays a role in stimulating IL-6 production *in vivo* in the CDE model but not in the DDC model. Finally, a previous report showed that *in vivo* treatment with IFN- γ enhanced hepatic inflammation and LPC growth in CDE-fed mice [14]. Taken together, in the CDE model, IFN- γ likely directly inhibits LPC proliferation, but it also indirectly enhances LPC expansion by promoting macrophage activation. The net effect of IFN- γ on LPC proliferation in the CDE model may be determined by the balance between these aforementioned responses.

Although the *in vitro* co-culture experiments in Fig. 4 suggest that IFN- γ may indirectly regulate LPC proliferation by targeting HSCs or macrophages, we did not prove this indirect effect *in vivo*. Further studies using cell-specific IFNGR KO or cell-specific STAT1 KO mice are required to tease out the cell-type dependent effect of IFN- γ on LPC proliferation *in vivo*.

Clinical significance of this study: The results from experimental DDC and CDE models and *in vitro* cell culture models suggest that IFN- γ can directly inhibit LPC proliferation but may also indirectly affect LPC growth by targeting HSCs and macrophages. Thus, the exact effects of IFN- γ on LPC proliferation in patients with chronic liver diseases may depend on the type, stage, and severity of liver diseases. For example, chronic HBV infection is associated with marked activation of adaptive immunity; such activation is not observed in both DDC and CDE models. Therefore, IFN- γ may also indirectly affect LPC proliferation in HBV patients by regulating adaptive immune responses. Future studies to identify these effects may help us understand better the effects of inflammation on LPC expansion and liver repair in patients with chronic liver diseases.

Figure 1. IFN- γ treatment reduces the number of LPCs and DRs in HBV patients and in DDC-treated mice. (A) CK19 and IFN- γ staining were performed using liver tissue from 30 HBV patients before IFN- γ treatment. Representative pictures show positive CK19 and negative IFN- γ staining patterns in these patients. (B) Representative images show CK19 staining in the biopsied liver tissue from HBV patients before and after IFN- γ treatment. (C, D) The number of liver LPCs and DRs was quantified from 18 HBV patients before and after IFN- γ treatment. * $P < 0.05$. (E) Representative H&E, Sirius red, α -SMA, CK19, and PCNA staining of liver tissues from mice fed a DDC diet for 2 weeks with/without IFN- γ treatment. (F) The degrees of inflammation and fibrosis, as well as the number of α -SMA⁺ cells, were quantified. (G, H) The numbers of CK19⁺ and PCNA⁺ cells from panel E were quantified. ** $P < 0.01$.

Figure 2. Disruption of the IFN- γ downstream signaling pathways increases liver inflammation, fibrosis, and LPC proliferation in DDC-challenged mice. Wild-type and genetically modified mice were fed a DDC diet for 2-4 weeks. (A-C) Representative images of Sirius red, α -SMA, and H&E staining from 2-week fed mice are shown on the left. The areas of Sirius red staining, α -SMA staining, and inflammation were quantified and are shown on the right. (D) Representative images of CK19 staining from DDC-exposed mice. (E) The areas of CK19⁺ staining from panel D were quantified. In panels A and E, * $P < 0.05$; ** $P < 0.01$ in comparison with the corresponding wild-type mice.

Figure 3. IFN- γ -mediated inhibition of LPC proliferation is STAT1- and IRF-1-dependent. (A) BrdU and pan-CK co-staining was performed in liver tissues from wild-type, STAT1^{-/-} and IRF-1^{-/-} mice fed a DDC diet for 4 weeks. The number of BrdU⁺pan-CK⁺ LPCs is shown in the right panel. * $P < 0.05$; ** $P < 0.01$ in comparison with WT group. (B) A BrdU incorporation assay was performed in IFN- γ -treated BMOL cells. The number of BrdU⁺ cells is shown in the right panel. (C) Western blot analysis of IFN- γ -treated BMOL cells. (D) Primary LPCs were isolated from mice fed a DDC diet for 4 weeks and treated with IFN- γ for 48 hours. BrdU and pan-CK co-staining was performed to measure LPC proliferation. The number of BrdU⁺pan-CK⁺

LPCs is shown in the right panel. In panels A, B, C, $*P<0.05$, $**P<0.01$, $***P<0.001$ in comparison with the group without IFN- γ treatment.

Figure 4. IFN- γ indirectly regulates LPC proliferation by targeting macrophages or HSCs. (A) Wild-type and IFN- $\gamma^{-/-}$ mice were fed a CDE diet for 4 weeks, and liver tissues were harvested for CK19 staining. The areas of CK19 $^{+}$ staining were quantified. (B) Macrophages were isolated from the peritoneal cavity and treated with IFN- γ for 6 hours. The relative gene expression was measured by real-time PCR. (C, D) Wild-type and IFN- $\gamma^{-/-}$ mice were fed a DDC or CDE diet for 4 weeks, and liver tissues were harvested for real-time PCR analysis. (E) Purified IFNGR1 $^{-/-}$ LPCs were co-cultured with intrahepatic lymphocytes (IHLs), macrophages, or HSCs with or without IFN- γ . LPC proliferation was measured by pan-CK and BrdU double staining. $*P<0.05$; $**P<0.01$. (F) **A schematic model depicting the direct and indirect effects of IFN- γ on LPC proliferation.** IFN- γ directly inhibits LPC proliferation via STAT1- and IRF-1-dependent mechanisms. IFN- γ may also indirectly suppress LPC proliferation by attenuating HSC activation, or it may indirectly promote LPC proliferation by inducing macrophage activation. The main difference between the DDC and CDE models is that HSC activation is predominantly associated with LPC proliferation in the DDC model, while macrophage activation is mainly involved in LPC proliferation in the CDE model.

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