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IL-37 suppresses the sustained hepatic IFN-γ/TNF-α production and T cell-dependent liver injury

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ABSTRACT

T cell-dependent liver injury is an important reason for the massive hepatic damage and cirrhosis. So far it is unclear whether the development of the disease could be efficiently suppressed by anti-inflammatory cytokine that modulates innate immune cells. Here we report that anti-inflammatory cytokine IL-37 could efficiently suppress the sustained hepatic expression of IFN-γ and TNF-α, two critical cytokines for inducing hepatocyte apoptosis and liver fibrosis in T cell-dependent liver injury. IL-37 could directly suppress IFN-γ/TLR4 ligand-induced M1 activation of macrophages, thus reducing the expression of pro-inflammatory cytokines TNF-α, IL-1β, and IL-12. Moreover, IL-37 attenuated Th1 response *in vivo* and increased the expression of Th2 cytokines IL-4 and IL-13, which in turn promoted M2 activation of macrophages in the liver. The increase of M2 activation not only further reduced TNF-α, IL-1β and IL-12 expression, but also increased IL-10 and IL-1Ra expression in macrophages, thus more efficiently suppressing the hepatic IFN-γ expression. By suppressing IFN-γ/TNF-α expression, IL-37 suppressed the up-regulation and activation of MLKL that drives hepatocellular necrosis in T cell-dependent liver damage. Accordingly, IL-37 efficiently reduced liver injury and hepatic inflammation after the repeated ConA challenge and the induction of autoimmune hepatitis, and also suppressed hepatic fibrosis resulting from the sustained liver damage. This study showed that the direct and indirect effect of IL-37 on macrophages could reduce the hepatic TNF-α expression, and also modulate IL-1β/IL-12 and IL-10/IL-1Ra expression to suppress the hepatic IFN-γ expression, thus suppressing the development of T cell-dependent liver injury such as autoimmune hepatitis.

1. Introduction

Liver damage occurring as a consequence of T cell activation is a serious health problem worldwide [1]. The most common causes of life-threatening T cell-dependent liver injury in humans are autoimmune hepatitis (AIH) and the infections with hepatitis B or C viruses [1]. The consequences of T cell-dependent liver injury, especially autoimmune liver destruction, are often severe, including massive hepatic damage, cirrhosis, and subsequent multiorgan dysfunction [2].

Many types of cytokines are involved in T cell-dependent liver injury [3–6]. Among them, IFN-γ and TNF-α play a critical role in disease development [5–7], since they are critical for inducing hepatocyte apoptosis and hepatic fibrosis in T cell-dependent liver injury [7,8]. Intriguingly, T cell-dependent liver injury mainly results from the intrahepatic production of IFN-γ and TNF-α, whereas the increase of plasma IFN-γ and TNF-α could not result in the damage of the liver [1]. Therefore, the control of intrahepatic production of IFN-γ and TNF-α might be crucial for suppressing T cell-dependent liver injury.

IFN-γ and TNF-α could be produced by T cells and macrophages respectively. Intriguingly, the production of these cytokines is related to the activation phenotype of macrophages. During inflammatory process, macrophages may undergo classical M1 activation (pro-inflammatory) or alternative M2 activation (anti-inflammatory) [9–11]. M2 macrophages are inefficient in producing TNF-α [12,13]. M1 macrophages produce TNF-α [14–16], and they are the main source of hepatic TNF-α [1]. On the other hand, the expression of IFN-γ by T cells could be regulated by macrophages. M1 macrophages produce IL-12 and IL-1β that promote the production of IFN-γ by T cells [16–18]. In contrast, M2 macrophages are inefficient in producing IL-12 and IL-1β [12,13], whereas produce IL-10 and interleukin-1 receptor antagonist (IL-1Ra) [12,13] that suppress the expression and secretion of IFN-γ by T cells [18–20]. It has been reported that the intrahepatic production of

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TNF-α and IFN-γ is mainly determined by the macrophages in the liver [1,15]. In this regard, the cytokine that modulates the activation of macrophages might be crucial for reducing the production of IFN-γ and TNF-α during T cell-dependent liver injury.

The previous studies showed that IL-37, a fundamental inhibitor of innate immunity [21], could suppress post-receptor signal transduction in macrophages, thus suppressing the expression of pro-inflammatory cytokines [21,22]. Moreover, the accumulated data reveal IL-37 as a natural modulator of inflammation to overcome an exacerbated immune reaction [21]. These findings suggest that IL-37 might have the potential to suppress T cell-dependent liver injury. However, it is unclear whether and how IL-37 could efficiently control the intrahepatic production of TNF-α and IFN-γ. Therefore, in this study we focused on the effect of IL-37 on the intrahepatic production of TNF-α and IFN-γ to gain the understanding of the influence of IL-37 on T cell-dependent liver injury, thus exploring the potential strategy for the therapy of T cell-dependent liver injury. For this purpose, we increased the expression of IL-37 in the liver of mice by transgene expression, and applied the model of T cell-dependent liver injury resulting from autoimmune hepatitis and ConA challenge, the animal model of T cell-dependent liver injury [1,23,24], to analyze the effect of IL-37 and the underlying mechanisms.

2. Materials and methods

2.1. Reagents

Concanavalin A (ConA) was purchased from Merck Millipore. Recombinant human IL-37 was purchased from R&D Systems. Murine IFN-γ, TNF-α, and M-CSF were purchased from PeproTech (Rocky Hill, NJ). Lipopolysaccharide (LPS) was purchased from Sigma-Aldrich (St. Louis, MO). Adenovirus (Ad) was purchased from Viraltherapy Technology Co., Ltd. (Wuhan, China).

2.2. In vivo gene transfection

Plasmids pIL37 and pCYP2D6 are expression vectors carrying the cDNA encoding human IL-37 and cytochrome P450 2D6 (CYP2D6) respectively. These plasmids were constructed by the insertion of cDNA into plasmid pcDNA3.1 (Invitrogen, Carlsbad, CA) in our laboratory. The plasmids were prepared and analyzed as described previously [25]. Mice received the injection of plasmid DNA *via* the tail vein (i.v. injection) using the hydrodynamics-based gene delivery technique [25], which delivers the transgene mainly into the liver [25,26].

2.3. Animal model and treatment

The animal model of T cell-dependent liver injury resulting from ConA challenge and autoimmune hepatitis (AIH) was used to analyze the effect of IL-37.

C57BL/6 mice, 7–8 weeks old, were purchased from Center of Medical Experimental Animals of Hubei Province (Wuhan, China) for studies. All animal experiments were approved by the Animal Care and Use Committee of Tongji Medical College. For ConA challenge, the mice received single intravenous (i.v.) injection, or the repeated i.v. injection, of ConA (15 mg/kg body weight per injection). To induce AIH, the mice received the i.v. injection of adenovirus (10^9 pfu) and pCYP2D6 plasmid (50 μg per injection). In this model, adenovirus was used to promote the induction of AIH by human CYP2D6 [2,27]. For the treatment with IL-37, the mice received the i.v. injection of pIL37 plasmid (200 μg per injection) to express IL-37 in the liver. Plasmid pcDNA3.1 was used as control. The mice were sacrificed at the indicated time points for the analysis of liver injury, histology, the activation phenotype of macrophages, and the gene expression in macrophages or liver tissues. The protocols for the injection of different agents are shown in Supplementary Fig. S1, and indicated in the corresponding figure legends.

2.4. Analysis of gene expression by conventional RT-PCR and real-time RT-PCR

Total RNA was extracted from cells or tissues with TRIzol reagent (Invitrogen, Carlsbad, CA). To identify the expression of transgene IL-37 in the liver, the mRNA of IL-37 was determined by RT-PCR (twenty-eight cycles, One-step RT-PCR kit, Qiagen, Valencia, CA), using β-actin mRNA as the internal control. The sequences of the primers used for detecting gene expression were as follows: IL-37, sense 5′-CTCTGCGG AGAAAGGAAGT-3′, antisense 5′-GCTGAAGGGATGGATGAC-3′; β-actin, sense 5′-ATGGGTCAGAAGGACTC CTATG-3′, antisense 5′-ATCTCCT GCT CGAAGTCTAGAG-3′.

The quantification of the expression of genes was performed using real-time RT-PCR as described previously [28]. The sequences of the primers used for detecting gene expression were as follows: *Mlkl*, sense 5′-AATTGTACTCTGGGAAATTGCCA-3′, antisense 5′-TCTC CAAGATTC CGTCCACAG-3′; *Ifng* (IFN-γ), sense 5′-ATG AACGCTACACACTGC ATC-3′, antisense 5′-CCATCCTTTTGCCAGTTCCTC-3′; *Tnfa* (TNF-α), sense 5′-CAGGCGGTGCC TATGTCTC-3′, antisense 5′-CGATCACCCCGA AGTTCAGTAG-3′; *Il1b* (IL-1β), sense 5′- TGGACCTTCCAGGATGAGGAC A-3′, antisense 5′-GTTCATCTCGGAGCCTGTAGTG-3′; *Il1rn* (IL-1Ra), sense 5′-GC TCATTGCTGGGTACTTACAA-3′, antisense 5′-CCAGACTTG GCACAAGACAG G-3′; *Il4* (IL-4), sense 5′-GGTCTCAACCCCCAGCT AGT-3′, antisense 5′-GCCGATGATCTCTCTCAAGTGAT-3′; *Il10* (IL-10), sense 5′-GCTCTTACTGACTG GCATG AG-3′, antisense 5′-CGCAGCTCT AGGAGCATGTG-3′; *Il12* (IL-12), sense 5′-CCAGGTGTCTTAGCCAG TCC-3′, antisense 5′-GCAGTGCAGGAATAATGTTTCA-3′; *Il13* (IL-13), sense 5′-CCTGGCTCTTGCTTGCCTT-3′, antisense 5′-GGTCTTGTGTG ATG TTGCTCA-3′; *Col1a1*, sense 5′-ATGGATTCCCGTTCGAGTACG-3′, antisense 5′-TC AGCTGGATAGCGACATCG-3′; *Col1a2*, sense 5′-CA CCCCAGCGAAGAACTCATA-3′, antisense 5′-GCCACCATTGATAGTCTC TCCTAAC-3′; *Nos2* (iNOS), sense 5′-GAAGA AAACCCCTTGTGCTG-3′, antisense 5′-TCCAGGGATTCTGGAACAT T-3′; *Arg1*, sense 5′-CTCCAA GCCAAAGTCCTTAGAG-3′, antisense 5′-AGGAGCTGTCATTAGGGACAT C-3′; *Ym1*, sense 5′-CAGGTCTGGCAATTCTTCTGAA-3′, antisense 5′-GTCTTGCTCATG TGTGTAAGTGA-3′; *Actb* (β-actin), sense 5′-AGGG AAATCGTGCGTGAC-3′, antisense 5′-CGCTCGTTGCCAATAGTG-3′. For sample analysis, the threshold was set based on the exponential phase of products, and C_T value for samples was determined. After analyzing the data with the comparative C_T method, the results were shown as the relative expression to housekeeping gene *Actb* [28]. For the purpose of showing the changes of different genes in the same panel in the figures, the expression levels of the genes were also shown as the fold change relative to control group which was designated as 1.

2.5. Western blot assay

Cells were treated with the indicated stimuli or isolated from mice. The cells were lysed, and the tissue samples after *in vivo* transfection were homogenized for Western blot as described previously [28,29]. Primary antibodies and horseradish peroxidase-conjugated secondary antibodies were purchased from StemCell Technologies (UK), Chemicon (Temecula, CA), and Santa Cruz (Santa Cruz, CA).

2.6. ELISA analysis

Cytokines in the supernatants of cell culture and the soluble interstitial molecules from the liver tissues (LT-SIM) were detected using ELISA kits. IL-37 was quantified using human IL-37 ELISA kit (AdipoGen, Switzerland). Other cytokines were detected using corresponding murine cytokine ELISA kits (R&D Systems, Minneapolis, MN; and Invitrogen, Carlsbad, CA). When cytokine in the culture supernatant was analyzed, the concentration (pg/ml) was determined. When cytokines in LT-SIM were detected, the cytokines were determined as

pg/mg protein. Based on the results of assays (pg/mg protein), the cytokine levels were also shown as the fold change relative to control group, for the purpose of showing the changes of different cytokines in the same panel in the figures.

To detect autoantibodies in serum, normal hepatic proteins were prepared by homogenizing the liver tissues from naive mice, followed by centrifugation. The proteins (500 μg/ml) were used to coat 96-well microtiter plates. Sera were added in PBS containing 2% FCS. The dilution series for each serum started at 1:100, followed by 1:2 dilution steps. Sera dilutions with values of three standard deviations above the mean value of negative controls were considered positive [2].

2.7. Analysis of liver injury

The extent of liver injury was assessed by the measurements of serum liver enzyme at the indicated time point after ConA administration or the induction of AIH. Whole blood was collected by orbital sinus puncture. Sera were collected after centrifugation and stored at −80 °C until analysis. The serum levels of alanine aminotransferase (ALT) were determined by ALT activity assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) to evaluate the hepatic injury.

2.8. Histology

Liver tissues from median and left lobes were collected, and embedded in paraffin according to standard histological procedures. Tissue sections were prepared and subjected to H&E staining for observation under a light microscope. An inflammation score was performed as previously described [26,30]. For lobular inflammation, no inflammation was counted as 0, mild lobular inflammation (< 10% of liver parenchyma) as 1, moderate lobular inflammation (10–50% of liver parenchyma) as 2, and a score of 3 was given for severe lobular inflammation (> 50% of liver parenchyma). For portal inflammation, no portal inflammation was counted as 0, mild portal inflammation (< 1/3 of portal tracts) as 1, moderate portal inflammation (approximately 1/2 of portal tracts) as 2, and a score of 3 was given for severe portal inflammation (> 2/3 of portal tracts). The scores for portal and lobular inflammation were added, representing the inflammation score.

2.9. Isolation and treatment of hepatocytes and hepatic macrophages

Hepatocytes were prepared from liver by two-step collagenase perfusion technique [31]. Hepatocytes were identified according to the size of the cells (Supplementary Fig. S2A and Ref. [32]). The isolated cells were > 93% hepatocytes. The cells were then used for the assay of gene expression, or cultured at a concentration 5×10^5 cells/ml.

The hepatic macrophages were separated from hepatocytes and other sinusoidal cells by gradient centrifugation as described previously [32]. Then, the method of selective adherence to plastic was used to purify macrophages [32,33]. After 2-h culture for adherence, macrophages were detached by incubation with 0.25% trypsin for 5 min, and pelleted by centrifugation [32]. The isolated cells were > 92% macrophages as assessed by flow cytometric analysis (Supplementary Fig. S2B) using FITC-anti-F4/80 antibody (eBioscience). The cells were then used for the assay of gene expression, or cultured at a concentration 1×10^6 cells/ml.

2.10. Preparation of soluble molecules from liver tissues

The mixture of soluble interstitial molecules from liver tissues (LT-SIM) was prepared by digesting the tissues with collagenase and removing debris by centrifugation. The concentration of LT-SIM was defined by the concentration of protein, which was determined by using Coomassie Bradford reagent (Thermo Fisher Scientific, Rockford, IL) according to the manufacturer's instructions.

2.11. Generation of murine bone marrow-derived macrophages

Murine bone marrow derived macrophages were obtained according to the protocol in previous publication [34]. Bone marrow cell suspensions were cultured in the DMEM medium supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycin and 10 U/ml recombinant murine M-CSF. Macrophages were then harvested on day 7 for subsequent cell stimulation assays.

2.12. Isolation of hepatic $CD4^+$ T cells

Non-parenchymal cells were prepared from the liver by gradient centrifugation as described previously [35]. The non-parenchymal cells were labeled with PE-conjugated anti-CD4 (eBioscience, San Diego, CA). The $CD4^+$ T cells were sorted with very stringent gating conditions (FACSAriaII cell sorter, BD Bioscience, San Jose, CA). The sorted cells used for the experiments were > 97% to 98% in purity which was checked by flow cytometry.

2.13. Cytokine neutralization

For IL-4 neutralization in the model of the sustained liver injury, the mice received the intraperitoneal injection of anti-IL-4 antibody (clone 11B11; BioXCell), 100 μg per injection. In ConA-model, anti-IL-4 antibody was injected on d1 and d3. In AIH model, anti-IL-4 antibody was injected from d33 to d45, once every three days. Control mice received equal amounts of isotype control antibody or equal volume of PBS.

2.14. Analysis of liver fibrosis

The liver tissues were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned at 5-μm thickness. Picrosirius staining was performed for detection of fibrosis. For quantitative assessment of fibrosis, a fibrosis score was performed by using a 0–4 scale: 0, no fibrosis; 1, minimal portal fibrosis; 2, portal fibrosis with septa formation; 3, localized bridging fibrosis; and 4, extensive bridging fibrosis [36]. Meanwhile, the expression of *Col1a1* and *Col1a2* genes was detected by real-time RT-PCR.

2.15. Statistical analysis

Results were expressed as mean value ± SD and interpreted by one-way ANOVA with Scheffe's multiple comparison test as *post hoc* test. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. IL-37 suppresses the sustained intrahepatic production of IFN-γ/TNF-α resulting from ConA and AIH

To investigate whether IL-37 could modulate the intrahepatic production of IFN-γ and TNF-α, and suppress T cell-dependent liver injury, we first used ConA to induce the liver injury (Supplementary Fig. S1A). IL-37 was expressed in the liver by *in vivo* transfection (Fig. 1A and Supplementary Fig. S1A), and mainly expressed in hepatocytes (Supplementary Fig. S3). Within the first 24 h after ConA injection, the transgene expression of IL-37 in the liver partially suppressed the up-regulation of *Ifng* (IFN-γ) and *Tnfa* (TNF-α) genes (Fig. 1B and Supplementary Fig. S4A). Accordingly, IL-37 only partially hindered the increase of ALT in serum (Fig. 1C). Intriguingly, if the mice were treated by the repeated ConA challenge (Supplementary Fig. S1B) to induce the sustained liver damage (Fig. 1D and Ref. [3]), IL-37 could efficiently suppress the increase of serum ALT level (Fig. 1D) and the hepatic inflammation (Supplementary Fig. S4B and S4C) after the repeated ConA challenge. In line with this, IL-37 efficiently suppressed the hepatic expression of *Ifng* and *Tnfa* genes (Fig. 1E and Supplementary Fig. S4D).

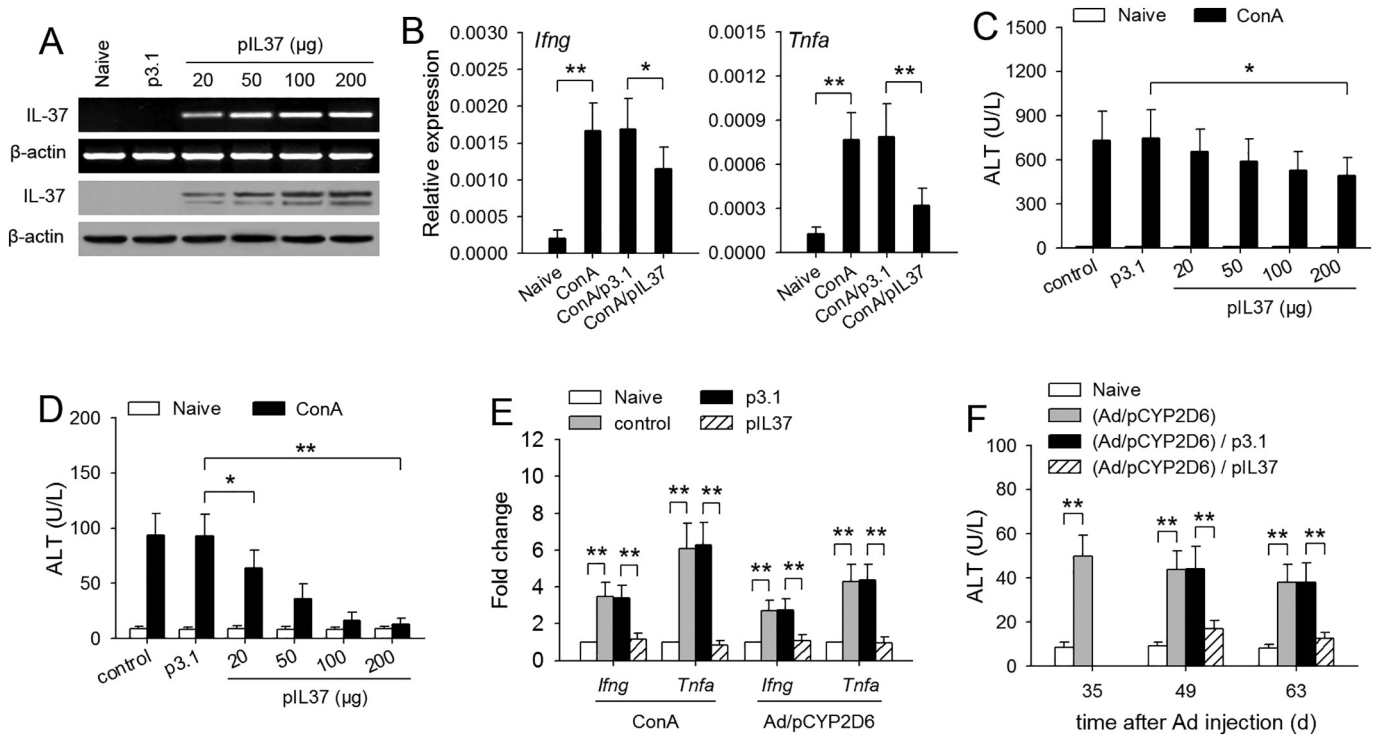

Fig. 1. IL-37 suppresses the sustained hepatic production of IFN-γ/TNF-α and liver damage. (A) The transgene expression of IL-37 in the liver was detected by RT-PCR and Western blot 48 h after i.v. injection of plasmid pIL37 with the indicated dosage. Plasmid pcDNA3.1 (p3.1, 200 μg per injection) was used as control. (B) Mice (n = 6 per group) received a single ConA challenge and the i.v. injection of pIL37 (see Fig. S1A). 16 h after the injection of ConA, the expression of *Ifng* and *Tnfa* genes in the liver tissues was detected at mRNA level by real-time RT-PCR. (C and D) Mice (n = 6 per group) received a single ConA challenge (C, also see Fig. S1A) or the repeated ConA challenge (D, also see Fig. S1B), with or without the i.v. injection of pIL37. The serum levels of ALT were detected 24 h after ConA challenge (C) or on d5 after the first ConA challenge (D). (E) The mice (n = 6 per group) received the repeated i.v. injection of ConA and pIL37 (see Fig. S1B) or the i.v. injection of adenovirus (Ad)/pCYP2D6 and pIL37 (see Fig. S1C). On d5 (ConA) or d49 (Ad/pCYP2D6), the expression of *Ifng* and *Tnfa* genes in the liver tissues was detected at mRNA level by real-time RT-PCR. (F) The mice (n = 6 per group) received the i.v. injection of Ad/pCYP2D6 with or without the i.v. injection of pIL37 (see Fig. S1C and S1D). The serum levels of ALT were detected on d35, d49 and d63 respectively. Plasmid pcDNA3.1 (p3.1) was used as control in the above experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

Furthermore, we induced autoimmune hepatitis (AIH) in mice and treated the mice by transgene expression of IL-37 in the liver (Supplementary Fig. S1C and S1D). When AIH was induced by Ad/CYP2D6, the liver injury was increased after Ad-induced injury disappeared, which was in accordance with the increase of Ad/CYP2D6-induced Th1 response in the liver (Fig. S5). After the induction of AIH, the transgene expression of IL-37 in the liver could suppress the hepatic expression of *Ifng* and *Tnfa* genes (Fig. 1E and Supplementary Fig. S6A). Consistently, IL-37 efficiently suppressed the liver injury (Fig. 1F) and the hepatic inflammation (Fig. S6B–D) resulting from AIH.

Taken together, these results suggest that IL-37 might not be very efficient in suppressing the hepatic production of IFN-γ and TNF-α during the initial ConA-induced liver injury, but IL-37 was efficient in suppressing the sustained hepatic production of IFN-γ and TNF-α, and suppressing T cell-dependent liver injury resulting from the repeated ConA challenge or AIH.

3.2. IL-37 modulates the hepatic expression of IL-1β/IL-12 and IL-10/IL-1Ra

To investigate whether IL-37 might influence macrophages to modulate the expression of IFN-γ by T cells, we analyzed the intrahepatic production of IL-1β/IL-12 and IL-10/IL-1Ra. 24 h after single ConA injection, the transgene expression of IL-37 in the liver attenuated the up-regulation of *Il1b* (IL-1β) and *Il12* (IL-12) genes that promote IFN-γ expression by Th1 cells (Fig. 2A and Supplementary Fig. S7A), but did not increase the expression of *Il10* (IL-10) and *Il1rn* (IL-1Ra) genes that suppress IFN-γ expression (Fig. 2B and Supplementary Fig. S7B). Interestingly, when the sustained liver injury resulted from the repeated ConA challenge, IL-37 not only suppressed the up-regulation of *Il1b* and *Il12* genes (Fig. 2C and Supplementary Fig. S7C), but also increased the expression of *Il10* and *Il1rn* genes (Fig. 2D and Supplementary Fig. S7D). Moreover, after the induction of AIH, the transgene expression of IL-37 in the liver could suppress the hepatic expression of *Il1b* and *Il12* genes (Fig. 2C and Supplementary Fig. S7C), and increase the expression of *Il10* and *Il1rn* genes (Fig. 2D and Supplementary Fig. S7D). These results indicate that the modulatory effect of IL-37 on the intrahepatic expression of IL-1β/IL-12 and IL-10/IL-1Ra was consistent with its effect on the hepatic expression of IFN-γ shown in Fig. 1.

3.3. IL-37 modulates macrophages to reduce hepatic TNF-α and IFN-γ expression

Next, we further investigated the effect of IL-37 on gene expression in the hepatic macrophages. During the first 24 h after ConA injection, the expression of *Tnfa*, *Il1b*, and *Il12* genes was increased in hepatic macrophages (Fig. 3A). The transgene expression of IL-37 in the liver attenuated the up-regulation of these genes, but did not increase the expression of *Il10* and *Il1rn* genes (Fig. 3A). Intriguingly, during the sustained liver injury resulting from ConA or AIH, the transgene expression of IL-37 in the liver not only more efficiently suppressed the up-regulation of *Tnfa*, *Il1b*, and *Il12* genes, but also increased the expression of *Il10* and *Il1rn* genes in hepatic macrophages (Fig. 3B). The effect of IL-37 on the expression of these genes in hepatic macrophages was consistent with its effect on the intrahepatic expression of these genes shown in Fig. 2.

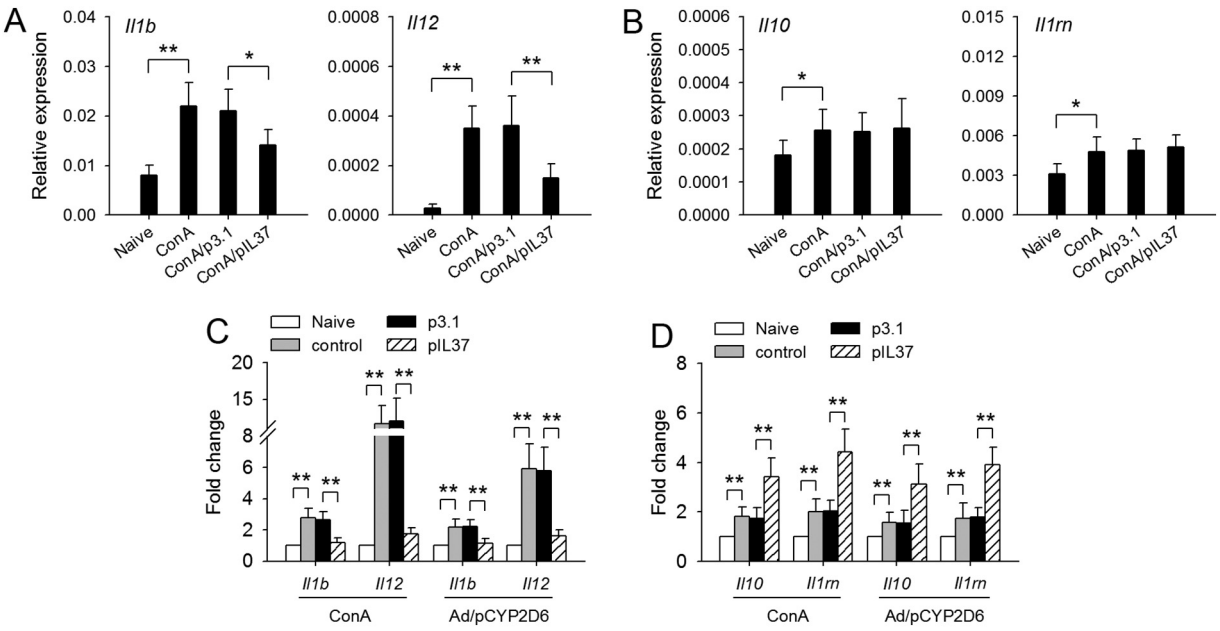

Fig. 2. IL-37 reduces the hepatic expression of *Il1b/Il12* genes and increases the sustained hepatic expression of *Il10/Il1rn* genes. (A and B) Mice (n = 6 per group) received a single ConA challenge and the i.v. injection of pIL37 (see Fig. S1A). 16 h after the injection of ConA, the expression of *Il1b*, *Il12*, *Il10* and *Il1rn* genes in the liver tissues was detected at mRNA level by real-time RT-PCR. (C and D) The mice ($n = 6$ per group) received the repeated i.v. injection of ConA and pIL37 (see Fig. S1B) or the i.v. injection of Ad/pCYP2D6 and pIL37 (see Fig. S1C). On d5 (ConA) or d49 (Ad/pCYP2D6), the expression of *Il1b*, *Il12*, *Il10* and *Il1rn* genes in the liver tissues was detected at mRNA level by real-time RT-PCR. Plasmid pcDNA3.1 (p3.1) was used as control in the experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

To further analyze the effect of macrophages on the hepatic production of TNF-α and IFN-γ during the sustained liver injury, we depleted macrophages *in vivo* (Supplementary Fig. S8). Depleting macrophages (Mφ) abrogated the hepatic expression of *Tnfa* gene, similar to the effect of IL-37 (Fig. 3C). Differently, however, depleting macrophages was not as efficient as the transgene expression of IL-37 in suppressing hepatic *Ifng* gene expression (Fig. 3C). Consistently, depleting macrophages only resulted in the reduced hepatic expression of *Il1b* and *Il12* genes (Fig. 3D), but not the increase of hepatic expression of *Il10* and *Il1rn* genes (Fig. 3E). These results suggest that IL-37 could modulate macrophage activation to reduce the hepatic expression of TNF-α and IFN-γ, and that both the suppression of *Il1b/Il12* up-regulation and the increase of *Il10/Il1rn* expression are important for the efficient suppression of the hepatic IFN-γ expression.

3.4. IL-37 suppresses M1 activation of macrophages to reduce TNF-α and IFN-γ expression

We next investigated the effect of IL-37 on the activation of macrophages to further analyze the mechanisms underlying its effect on the hepatic production of TNF-α and IFN-γ. M1 activation results in the up-regulation of inducible nitric oxide synthase (iNOS) and TNF-α in macrophages, while M2 activation results in the increase of arginase 1 (Arg1) and Ym1 expression [9–11]. In the *in vitro* experiments, IL-37 could directly suppress M1 activation of macrophages induced by IFN-γ and LPS (a representative TLR4 ligand) (Fig. 4A). When naive macrophages were cultured in the presence of the mixture of soluble interstitial molecules from the liver tissues (LT-SIM), which might represent complex stimuli in the liver tissue, LT-SIM from ConA-mice induced M1 activation of macrophages, which was also suppressed by IL-37 (Fig. 4B). Accordingly, IL-37 suppressed IFN-γ/LPS- or LT-SIM(ConA-mice)-induced expression of *Il1b* and *Il12* genes (Fig. 4C). However, IL-37 did not promote M2 activation of macrophages (Fig. 4A and B), and accordingly, did not increase the expression of *Il10* and *Il1rn* genes (Fig. 4C). Therefore, by suppressing M1 activation, IL-37 can only partially reduce the production of TNF-α and IFN-γ.

3.5. IL-37 indirectly promotes M2 activation of macrophages to further reduce TNF-α and IFN-γ expression

In line with the results of *in vitro* experiments shown in Fig. 4, the transgene expression of IL-37 *in vivo* suppressed M1 activation of intrahepatic macrophages, but did not promote M2 activation during the first 24 h after ConA injection (Supplementary Fig. S9A). Intriguingly, however, IL-37 could promote M2 activation of hepatic macrophages during the sustained liver injury resulting from ConA (Fig. 5A) and AIH (Supplementary Fig. S9B). These results were consistent with the effect of IL-37 on the expression profile of the genes in hepatic macrophages shown in Fig. 3A and B. Moreover, IL-37 could not directly promote M2 activation of macrophages, but LT-SIM from ConA/pIL37-mice and AIH/pIL37-mice could induce M2 activation of macrophages (Supplementary Fig. S9C and S9D), suggesting that the transgene expression of IL-37 in the liver could indirectly promote M2 activation of macrophages to further reduce the hepatic expression of TNF-α and IFN-γ.

We then focused on the sustained liver injury to analyze Th1 and Th2 responses, since Th2 cytokine IL-4 and IL-13 have been known to induce M2 activation of macrophages [12]. In the liver, ConA induced Th1-biased response, characterized by the increased mRNA levels of Th1 cytokine genes *Il2* (IL-2) and *Ifng* in the hepatic $CD4^+$ T cells, whereas ConA/pIL37 induced Th2-biased response, characterized by the increased expression of *Il4* (IL-4) and *Il13* (IL-13) genes (Fig. 5B and Supplementary Fig. S10A). Similar effect of IL-37 on Th1/Th2 responses was observed in AIH mice (Supplementary Fig. S10B), although IL-37 did not significantly increase the serum levels of autoantibodies (Supplementary Fig. S10C).

We next blocked IL-4 with neutralizing antibody *in vivo*. When IL-4 was blocked, the transgene expression of IL-37 *in vivo* could not efficiently promote M2 activation of hepatic macrophages (Fig. 5C and Supplementary Fig. S11A), and was inefficient in increasing the expression of *Il10* and *Il1rn* genes (Fig. 5D and Supplementary Fig. S11B). The same results were obtained when macrophages were stimulated *in vitro* with LT-SIM(ConA/pIL37) in the presence of anti-IL-4 neutralizing antibody (Fig. 5E and F). Taken together, these results suggest that IL-37 could promote Th2-biased response during the sustained

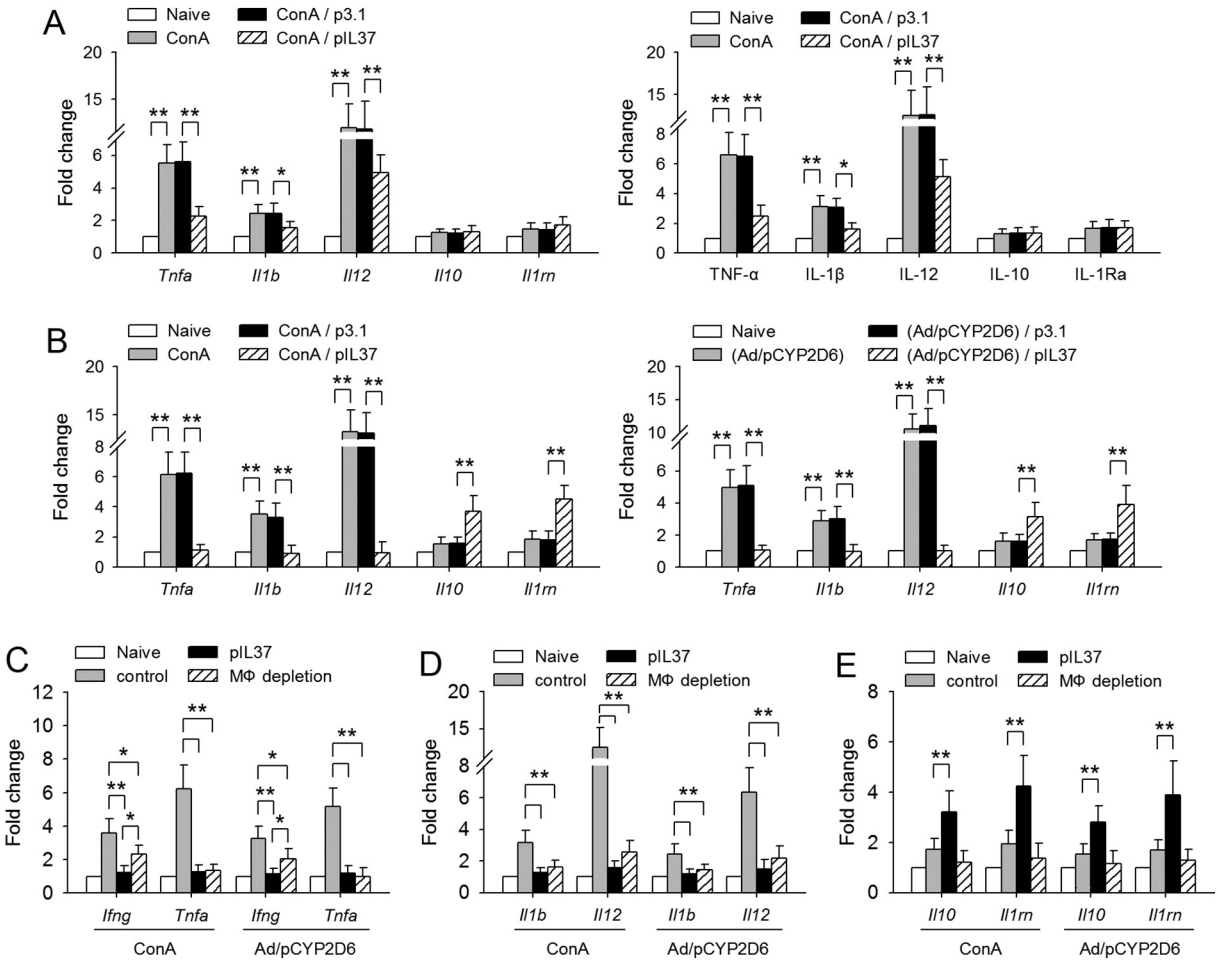

Fig. 3. IL-37 modulates the cytokine gene expression of hepatic macrophages *in vivo*. (A) Mice ($n = 6$ per group) received a single ConA challenge and the i.v. injection of pIL37 (see Fig. S1A). 16 h after the injection of ConA, macrophages were isolated from the liver. The expression of *Tnfa*, *Il1b*, *Il12*, *Il10*, and *Il1rn* genes in the macrophages was detected by real-time RT-PCR after isolation (left). After 48-h culture of the cells, the cytokines in the supernatants were detected by ELISA (right). (B) The mice (n = 6 per group) received the repeated i.v. injection of ConA and pIL37 (see Fig. S1B) or the i.v. injection of Ad/pCYP2D6 and pIL37 (see Fig. S1C). On d5 (ConA) or d49 (Ad/pCYP2D6), macrophages were isolated from the liver. The expression of *Tnfa*, *Il1b*, *Il12*, *Il10*, and *Il1rn* genes in the macrophages was detected by real-time RT-PCR. (C–E) The mice ($n = 6$ per group) received the repeated ConA injection (see Fig. S1B) or the i.v. injection of Ad/pCYP2D6 (see Fig. S1C). The mice also received the injection of pIL37 (see Fig. S1B and S1C) or Clip (see Fig. S8B and S8C). On d5 (ConA) or d49 (Ad/pCYP2D6), the expression of *Ifng*, *Tnfa*, *Il1b*, *Il12*, *Il10* and *Il1rn* genes in the liver tissues was detected at mRNA level by real-time RT-PCR. Plasmid pcDNA3.1 (p3.1) was used as control in the experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

inflammation, thus promoting M2 activation of macrophages to further reduce the hepatic production of TNF-α and IFN-γ.

3.6. IL-37 suppresses MLKL up-regulation and activation in hepatocytes

IFN-γ and TNF-α induce the hepatocellular necrosis in T cell-dependent liver damage by up-regulating and activating mixed lineage kinase domain-like protein (MLKL), a key factor in the cellular necrosome, in hepatocytes [7]. Based on the above results, we next focused on the sustained liver injury to analyze the effect of IL-37 on MLKL in hepatocytes. The expression and activation (phosphorylation) of MLKL in the hepatocytes was increased after ConA challenge, which was suppressed by IL-37 (Fig. 6A and B). The same results were obtained when AIH mice were treated by transgene expression of IL-37 in the liver (Fig. 6C and D). Intriguingly, however, when the hepatocytes from naive mice were stimulated with IFN-γ and TNF-α *in vitro*, both IFN-γ-induced up-regulation and TNF-α-induced activation (phosphorylation) of MLKL were not influenced by IL-37 (Fig. 6E and F). These results indicate that IL-37 could reduce the hepatic production of IFN-γ/TNF-α to protect hepatocytes from immune damage, but not directly antagonize the effect of IFN-γ/TNF-α.

3.7. IL-37 suppresses hepatic fibrosis resulting from AIH

In T cell-dependent liver injury, the sustained production of IFN-γ/TNF-α and the continuous presence of liver injury have been known to result in the hepatic fibrosis [8]. In AIH mice, the hepatic fibrosis could be efficiently suppressed by the transgene expression of IL-37 in the liver (Fig. 7A and B). Consistently, the up-regulation of *Col1a1* and *Col1a2* genes was suppressed by IL-37 (Fig. 7C). These results further indicate that IL-37 could efficiently suppress T cell-dependent liver injury.

4. Discussion

The severe consequences of T cell-dependent liver injury are mainly hepatocyte damage and hepatic fibrosis. The intrahepatic IFN-γ and TNF-α are crucial cytokines for inducing hepatocyte apoptosis and hepatic fibrosis in T cell-dependent liver injury [1,7,8]. Intriguingly, our data in this study showed that IL-37, a fundamental inhibitor of innate immunity, could efficiently suppress the sustained intrahepatic production of both IFN-γ and TNF-α through a regulatory network. A relatively longer period of time was required for IL-37 to trigger this

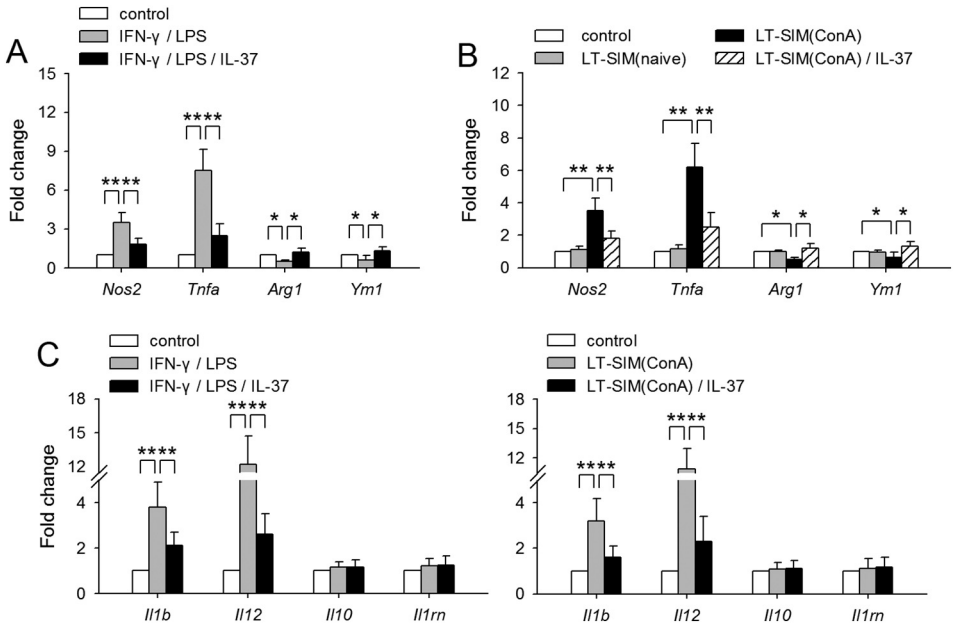

Fig. 4. IL-37 suppresses M1 activation of macrophages. (A–C) Macrophages were prepared from naive mice as described in Methods. The cells were stimulated with the indicated stimuli for 48 h. The expression of the indicated genes was detected by real-time RT-PCR. (A) The macrophages were unstimulated (control) or stimulated with IFN-γ (50 ng/ml)/LPS (100 ng/ml) in the absence or presence of IL-37 (100 ng/ml). (B) The macrophages were unstimulated or stimulated with LT-SIM from naive mice (LT-SIM(naive)) or ConA-mice (LI-SIM(ConA)) in the absence or presence of IL-37. (C) The macrophages were unstimulated or stimulated with IFN-γ/LPS or LT-SIM(ConA) in the absence or presence of IL-37. Data are pooled from three independent experiments with a total of six samples in each group. $^{*}P < 0.05$, $^{**}P < 0.01$.

regulatory network. Therefore, IL-37 was inefficient in suppressing the initial immune liver injury within a relatively short period of time, but was efficient in suppressing the sustained intrahepatic IFN-γ/TNF-α production and T cell-dependent liver injury.

The activation phenotype of macrophages is crucial for the hepatic production of not only TNF-α but also IFN-γ. In T cell-dependent liver injury, the hepatic macrophages mainly undergo M1 activation as shown by our data. M1 macrophages are the main source of hepatic TNF-α [1]. Moreover, M1 macrophages also produce IL-12 and IL-1β. IL-12 promotes the polarization of Th0 cells towards Th1 cells that produce IFN-γ [16,17]. IL-1β promotes the production of IFN-γ by T cells [18]. IL-37 could directly suppress macrophage activation in the presence of IFN-γ and TLR-4 ligand, the main stimuli to induce M1 activation of macrophages [12]. IL-37 suppresses the activation of signaling pathways including STATs 1–4, ERK and JNK, which are involved in the expression of TNF-α, IL-1 and IL-12 [21,22]. Inhibiting M1 activation of macrophages by IL-37 could not only reduce TNF-α in the liver, but also reduce IL-12 and IL-1β production to attenuate Th1 response, reducing the expression of Th1 cytokine IFN-γ. Therefore, the inhibitory effect of IL-37 on the hepatic production of IFN-γ and TNF-α could be partially explained by its inhibitory effect on M1 activation of macrophages. On the other hand, TLR4 ligands could be released from the damaged hepatocytes [37], since many intracellular molecules are TLR4 ligands, including HMGB1, HSP70, HSP60, S100A8, S100A9, and so on [38]. Reducing IFN-γ/TNF-α production resulted in the suppression of liver injury, which might further attenuate M1 activation of macrophages due to the reduced release of TLR4 ligands.

IL-37 could directly suppress M1 activation of macrophages, but it could not directly promote M2 activation of macrophages as shown by our data. Intriguingly, however, IL-37 could indirectly promote M2 activation of hepatic macrophages by influencing Th1/Th2 responses. Physiologically, Th1 and Th2 antagonize each other [17]. By suppressing M1 activation of macrophages, IL-37 could reduce the expression of IL-12 and IL-1β, thus attenuating Th1 response and favoring Th2 response, which was evidenced by the increased expression of Th2 cytokine IL-4 and IL-13. It has been known that IL-4 and IL-13 induce M2 activation of macrophages, which in turn exert immunoregulatory functions [12]. Indeed, the promoting effect of IL-37 on M2 activation of macrophages *in vivo* could be abrogated by blocking IL-4, confirming that IL-37 could indirectly promote M2 activation of macrophages by increasing the production of Th2 cytokines.

Increasing IL-4 production could negatively control IFN-γ production, since IL-4 can suppress the generation of Th1 cells by suppressing IL-12 signaling [39,40]. Moreover, increasing M2 activation of hepatic macrophages could more efficiently suppress hepatic production of IFN-γ and TNF-α. In contrast to M1 macrophages, M2 macrophages produce IL-10 and IL-1Ra, but not TNF-α, IL-1β and IL-12 [12]. IL-10 can suppress the expression of IFN-γ by T cells [19]. IL-1Ra could inhibit IL-1β signaling that is required for the secretion of IFN-γ from T cells [18,20]. IL-37 is an inhibitor of innate immunity [21], and mainly suppresses the expression of pro-inflammatory cytokines in macrophages [21,22]. By simply suppressing M1 activation of macrophages, IL-37 could partially reduce, but not completely suppress, the up-regulation of TNF-α, IL-1β, and IL-12, and could not increase IL-10 and IL-1Ra expression. Therefore, IFN-γ expression could not be efficiently suppressed. Consistently, during the initial inflammation induced by single ConA challenge, the transgene expression of IL-37 only partially suppressed liver damage. This is consistent with other reports that the initial (first 24 h) ConA-induced hepatocyte cell death could not be efficiently suppressed by anti-inflammatory cytokine [4,17]. Differently, during the sustained liver injury resulting from ConA and AIH, the up-regulation of TNF-α, IL-1β, and IL-12 in hepatic macrophages was completely suppressed if M2 activation was enhanced. IL-37 could not directly promote IL-10 and IL-1Ra expression in macrophages as shown by our data and others [21]. However, IL-10 and IL-1Ra expression was increased if the transgene expression of IL-37 enhanced M2 activation of hepatic macrophages, but could not be increased if M2 activation of hepatic macrophages was suppressed by blocking IL-4. Therefore, the enhancement of M2 activation of macrophages by transgene expression of IL-37 is crucial for more efficiently suppressing the hepatic production of IFN-γ and TNF-α.

In T cell-dependent liver injury such as autoimmune hepatitis (AIH) and ConA-induced hepatitis, hepatocellular necrosis is driven by MLKL-dependent pathway [7]. IFN-γ and TNF-α promote MLKL expression and its subsequent phosphorylation (activation) respectively, thus driving inflammation-dependent hepatocellular death [7]. Our data showed that IL-37 could not directly suppress IFN-γ-induced MLKL expression and TNF-α-induced MLKL activation, and therefore might not directly protect hepatocytes from IFN-γ/TNF-α-induced damage. Importantly, however, IL-37 could efficiently suppress the sustained hepatic production of IFN-γ and TNF-α. Thus, the transgene expression of IL-37 in the liver could efficiently suppress the up-regulation and activation of MLKL in the hepatocytes resulting from the repeated ConA challenge and AIH, thus protecting hepatocytes and preventing the

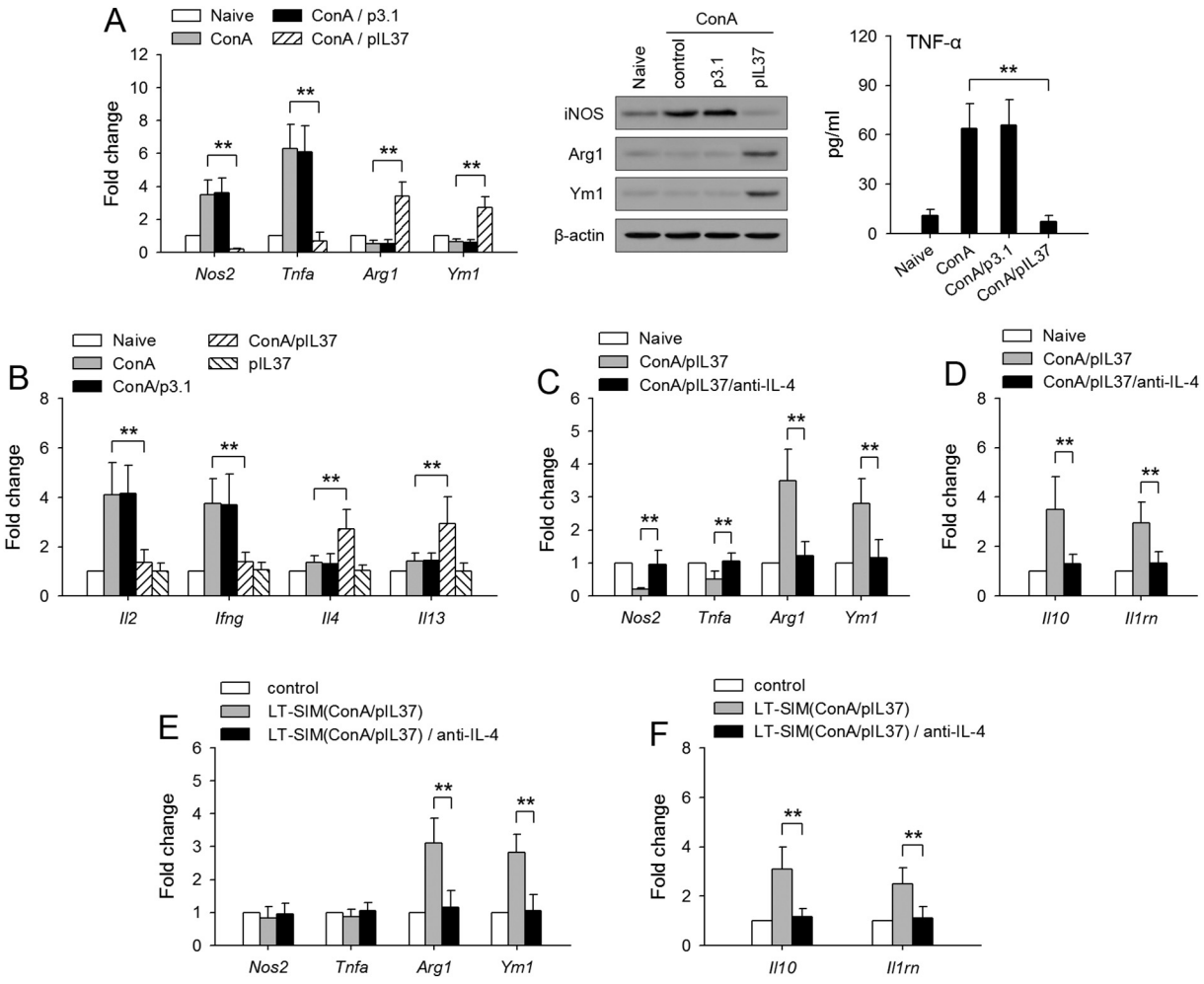

Fig. 5. IL-37 augments Th2 response to promote M2 activation of macrophages. (A) The mice ($n = 6$ per group) received the repeated i.v. injection of ConA and pIL37 (see Fig. S1B). On d5, macrophages were isolated from the liver. The expression of *Nos2*, *Tnfa*, *Arg1*, and *Ym1* genes was detected by real-time RT-PCR (left). iNOS, Arg1 and Ym1 were detected by Western blot (middle). After 48-h culture of the cells, TNF-α in the supernatants was detected by ELISA (right). (B) The mice (n = 6 per group) were untreated or received the repeated i.v. injection of ConA and pIL37 (see Fig. S1B). On d5, $CD4^+$ T cells were isolated from non-parenchymal cells of the liver. The expression of *Il2*, *Ifng*, *Il4* and *Il13* genes in the cells was detected at mRNA level by real-time RT-PCR. (C and D) The mice (n = 6 per group) were untreated or received the repeated i.v. injection of ConA and pIL37 (see Fig. S1B) with or without the i.p. injection of anti-IL-4 neutralizing antibody as described in Methods. On d5, macrophages were isolated from the liver. The expression of the indicated genes was detected by real-time RT-PCR. (E and F) The macrophages prepared from naive mice were unstimulated (control) or stimulated for 48 h with LT-SIM from ConA/pIL37-mice (see Fig. S9, figure legend) in the absence or presence of anti-IL-4 neutralizing antibody (10 μg/ml). The expression of the indicated genes was detected at mRNA level by real-time RT-PCR. $^{**}P < 0.01$.

hepatic fibrosis.

In summary, our data in this study showed that IL-37 could efficiently suppress the sustained intrahepatic production of IFN-γ and TNF-α through a regulatory network. IL-37 could directly suppress M1 activation of hepatic macrophages, which reduces IL-1β/IL-12 expression to attenuate Th1 response, favoring Th2 response. Th2 cytokines in turn promote M2 activation of hepatic macrophages, which not only further suppresses IL-1β and IL-12 expression, but also increases IL-10 and IL-1Ra expression, thus more efficiently suppressing the intrahepatic production of IFN-γ by T cells. Meanwhile, the suppression of M1 activation and the increase of M2 activation of macrophages results in the efficient suppression of TNF-α production in the liver.

Our findings in this way suggest that IL-37 could efficiently suppress T cell-dependent liver injury and the following hepatic fibrosis, and that modulating macrophage activation with IL-37 could be an important strategy for the therapy of T cell-dependent liver injury and the prevention of hepatic fibrosis. In addition to autoimmune hepatitis, the infections with hepatitis B or C viruses also result in T cell-dependent liver injury. In this situation, it should be taken into consideration whether and how the specific response against infection and the treatment protocols might influence the production and function of IL-37 or the activation of macrophages, which might be very important for the designing of the therapeutic strategy in the cases such as the infections with hepatitis B or C viruses. Moreover, in this study we mainly focused on T cell dependent liver injury. The model may not match with liver injury due to viral hepatitis or drugs. Further study will be needed to clarify the effect of IL-37 on the liver injury resulting from viruses and drugs.

Acknowledgment

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Disclosure of potential conflicts of interest

The authors declare that there are no conflicts of interest to disclose.

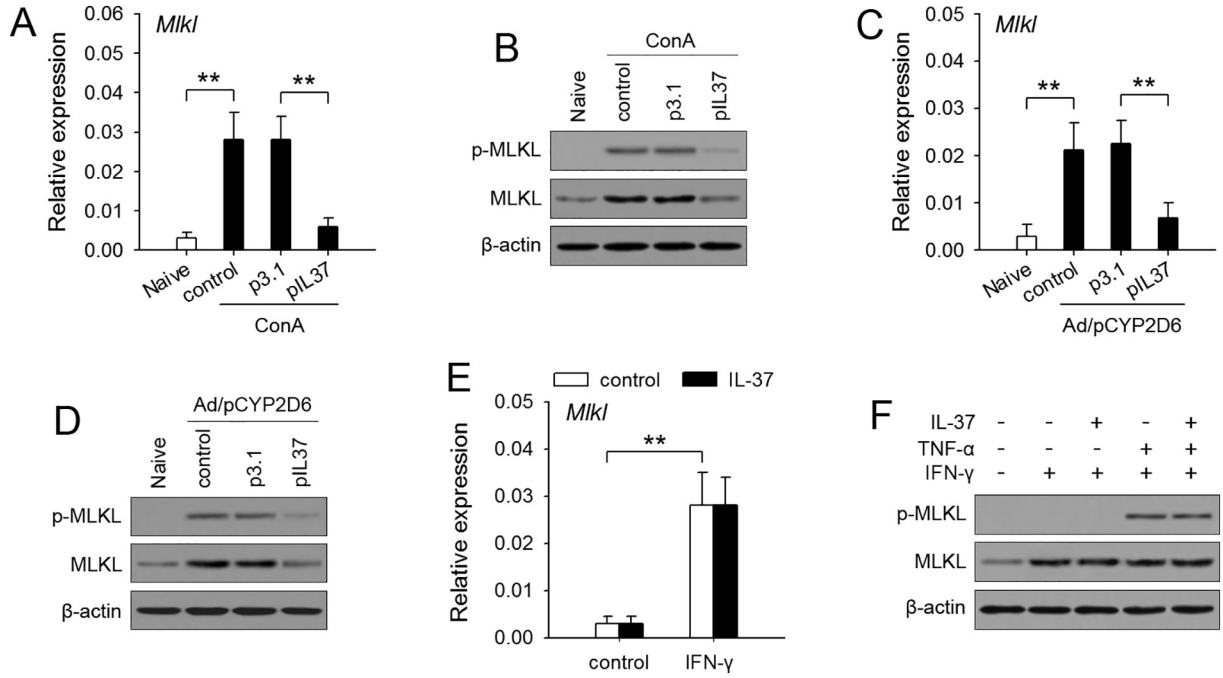

Fig. 6. IL-37 indirectly suppresses the up-regulation and activation of MLKL in hepatocytes *in vivo*. (A-D) The mice (n = 6 per group) were untreated or received the repeated i.v. injection of ConA and pIL37 (A, B, see Fig. S1B). or the i.v. injection of adenovirus (Ad)/pCYP2D6 and pIL37 (C, D, see Fig. S1C). Plasmid pcDNA3.1 (p3.1) was used as control. Hepatocytes were isolated from the mice on d5 (A, B) or d49 (C, D). The expression of *Mlkl* gene was detected by real-time RT-PCR (A, C).The MLKL and phospho-MLKL were detected by Western blot (B, D). (E and F) Hepatocytes were isolated from naive mice (n = 6 per group). The cells were cultured for 48 h in the absence or presence of IFN-γ (50 ng/ml), TNF-α (50 ng/ml), and IL-37 (100 ng/ml). The expression of *Mlkl* gene was detected by real-time RT-PCR (E). The MLKL and phospho-MLKL were detected by Western blot (F). $^{**}P < 0.01$.

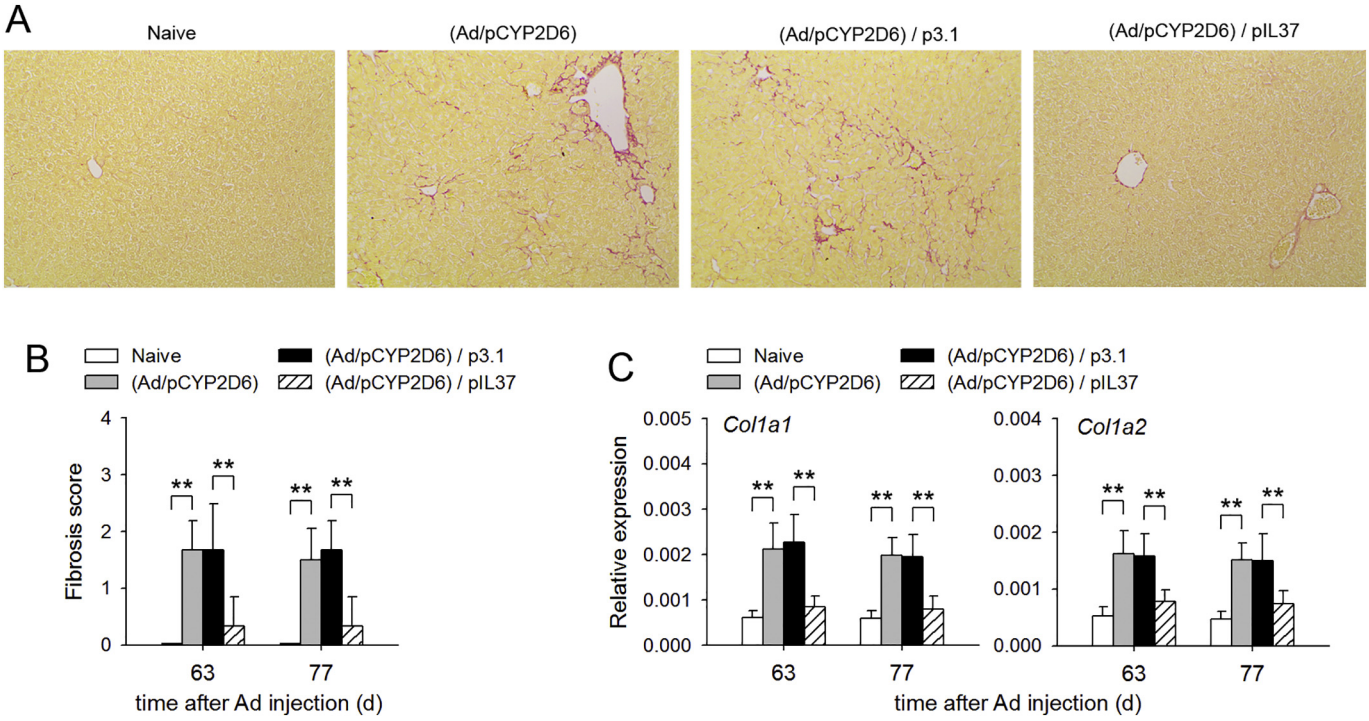

Fig. 7. IL-37 suppresses the liver fibrosis resulting from AIH. The mice (n = 6 per group) received the i.v. injection of adenovirus (Ad)/pCYP2D6 to induce AIH, and also received the i.v. injection of pIL37 (see Fig. S1D). Plasmid pcDNA3.1 (p3.1) was used as control. The fibrosis in liver tissues was detected by Picrosirius staining on d63 (A, B) and d77 (B). The expression of *Col1a1* and *Col1a2* genes in the liver was detected by real-time RT-PCR (C). $^{**}P < 0.01$.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.01.037.

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