### Title

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# Endogenous IL-10 Maintains Immune Tolerance but IL-10 Gene Transfer Exacerbates Autoimmune Cholangitis

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## Abstract

The immunomodulatory effect of IL-10 as an immunosuppressive and anti-inflammatory cytokine is well known. Taking advantage of our established mouse model of autoimmune cholangitis using 2-octynoic acid conjugated ovalbumin (2-OA-OVA) induction, we compared liver pathology, immune cell populations and antimitochondrial antibodies between IL-10 knockout and wild type mice immunized with 2-OA-OVA. At 10 weeks post immunization, portal inflammation and fibrosis were more severe in 2-OA-OVA immunized IL-10 knockout mice than in wild type mice. This was accompanied by significant higher levels of collagen I and III expression, T, NK and NKT subsets in liver and IgG anti-mitochondrial autoantibodies (AMA) compared to 2-OA-OVA immunized wild type mice, suggesting that endogenous IL-10 is necessary for the maintenance of immune tolerance in primary biliary cholangitis (PBC). Further, we investigated whether administration of exogenous IL-10 could prevent PBC by administration of IL-10 expressing recombinant adeno-associated virus (AAV-IL-10) either 3 days before or 3 weeks after the establishment of liver pathology. Interestingly, administration of AAV-IL-10 resulted in increased liver inflammation and fibrosis, accompanied by increases in IFN-γ in liver $CD4^+$ T cell, granzyme B, FasL, and CD107a in liver $CD8^+$ T and NKT cells, and granzyme B and FasL in liver NK cells of AAV-IL-10 administered mice compared with control mice. Furthermore, administration of AAV-IL-10 significantly increased levels of proinflammatory cytokines and chemokines (IFN-γ, TNF-α, CXCL9 and CXCL10) and collagen I and III production in naive mice, together with increase in immune cell infiltration and collagen deposition in the liver, suggesting a role of IL-10 in fibrosis. In conclusion, our data demonstrate that endogenous IL-10 is critical in the maintenance of immune tolerance but exogenous administration of IL-10

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exacerbates liver inflammation and fibrosis. Furthermore, the distinctive presence of inflammatory immune cell populations and collagen expression in AAV-IL-10 treated naive mice cautions against the clinical use of exogenous IL-10 in patients with autoimmune cholangitis.

**Keywords**

IL-10; liver autoimmune disease; adeno-associated virus; IFN-γ; inflammation

## 1. Introduction

Primary biliary cholangitis (PBC) is a progressive autoimmune liver disease by immune-mediated destruction of intrahepatic small bile ducts and characterized by anti-mitochondrial autoantibodies (AMA) and portal infiltrating T cells, both directed against the inner lipoyl domain of the E2 component of the pyruvate dehydrogenase complex (PDC-E2) [1, 2]. Despite intensive studies demonstrating a multilineage innate and adaptive immune response, there remains a major void in the treatment of patients [3–17].

IL-10 is predominantly an immunosuppressive and anti-inflammatory cytokine, generally considered a protective cytokine that facilitates immune regulation, secreted by a myriad of immune effector cells including helper and cytotoxic T cells, regulatory T cells, NK cells, NKT cells, monocytes, macrophages, dendritic cells, and granulocytes, i.e. neutrophils and eosinophils [18–22]. The IL-10 receptor, composed of *Il10ra* and *Il10rb,* is expressed on both innate and adaptive immune cells as well as non-hematopoietic cells [21]. IL-10 achieves suppression of inflammatory responses by inhibiting expression of MHC class II, costimulatory molecules, and proinflammatory cytokines in macrophages and dendritic cells, directly inhibiting the activation and cytokine secretion of $CD4^+$ T cells or promoting the maintenance, expansion and function of regulatory T cells [19, 21, 23, 24]. IL-10 can also be secreted by Kupffer cells, liver sinusoidal endothelial cells, and hepatic stellate cells [25, 26]. Liver IL-10 maintains tolerance of grafts and also promotes regulatory T cells [27]. Furthermore IL-10 downregulates the inflammatory response in liver injury [28, 29]. However HBV- and HCV-specific cytotoxic T cells are themselves capable of producing IL-10 and can thereby attenuate antiviral immunity via an autocrine feedback loop, further aggravating immune tolerance [30]. In addition, an antifibrotic activity of IL-10 has been demonstrated [28, 31–33]; IL-10 knockout mice have increased fibrosis in response to toxic injury [28] and transgenic IL-10 mice have decreased fibrosis using carbon tetrachloride ($CCl_4$) and thioacetamide-induced liver fibrosis models [34].

Given the powerful anti-inflammatory and anti-fibrotic properties of IL-10 in liver, we took advantage of our murine model of autoimmune cholangitis to ascertain the therapeutic potential of IL-10. We compared liver pathology, immune cell populations and AMAs between IL-10 knockout and wild type mice immunized with 2-OA-OVA. We intravenously injected mice either before or after the establishment of hepatic pathology with an IL-10 expressing recombinant adeno-associated virus (AAV-IL-10). We report herein that endogenous IL-10 is critical in the maintenance of immune tolerance but exogenous administration of IL-10 increases liver inflammation and fibrosis. Our data emphasize that

any immune therapeutic regimen that impacts IL-10 may be a two edge sword, potentially have both benefits and harm [35].

## 2. Materials and Methods

### 2.1 Experimental mice

IL-10 knockout mice on a C57BL/6 background were provided from Dr. Shau-Ku Huang (Johns Hopkins University, Baltimore, MD, United States). C57BL/6 mice were obtained from the National Laboratory Animal Center, Taiwan. All mice were maintained in the Animal Center of the College of Medicine, National Taiwan University. All experiments were performed following approval of The Institutional Animal Care and Use Committee (IACUC) of National Taiwan University College of Medicine and College of Public Health.

### 2.2 Experimental protocol

Female C57BL/6 mice or IL-$10^{-/-}$ female mice and age and sex-matched wild type littermates, at 7–9 weeks of age, were intraperitoneally immunized with 2-OA-OVA in the presence of complete Freund's adjuvant (CFA, Sigma-Aldrich, St. Louis, MO, USA) and subsequently boosted at weeks 2, 4, 6 and 8 with 2-OA-OVA in incomplete Freund's adjuvant (IFA, Sigma-Aldrich). Two μg of α-galactosylceramide (KRN7000, Cayman, Ann Arbor, MI, USA) were intravenously injected with the first and second 2-OA-OVA immunizations. AAV-IL-10 ($1\times10^7$ TU/mouse or $3\times10^7$ TU/mouse) was intravenously administered to mice at 3 days before or at 3 weeks after the first 2-OA-OVA immunization. Three weeks was chosen of this study because at 3 weeks following initial immunization with 2-OA-OVA, mice exhibit florid portal inflammation [36]. Sera were obtained on all mice at 10 weeks post-immunization and levels of IL-10 were measured by ELISA. Mice were sacrificed at 5 weeks post immunization for $CD4^+$ T cells, $CD8^+$ T cells, NK cells and NKT cells functional analysis, as described below. Additional mice were sacrificed at 10 weeks post-immunization for liver pathology, definition of mononuclear cell phenotypes, cytokine profiles, collagen levels and titers of AMAs. All experiments were performed a minimum of 2–4 times with group sizes of 5–11 mice.

### 2.3 Preparation of AAV-IL-10

IL-10 cDNA derived from activated mouse T cells was cloned into recombinant adeno-associated virus vector (pAAV-IRES-GFP) (Cell Biolabs, San Diego, CA, USA). IL-10 inserted pAAV-IRES-GFP plasmid was co-transfected with pAAV-DJ and pHelper at a ratio of 1:1:1 into the adenovirus packaging HEK293T cell line. Viruses were purified from infected cells 42–48 h after infection by three freeze-thaw cycles followed by Hi-Trap Heparin column. After concentration, viral titers (transduction unit, TU) were measured by GFP expression in infected HEK293T cells using flow cytometry. Throughout these studies a mock AAV was used as a control; it did not contain a transgene in the expression cassette.

### 2.4 Determination of serum AMAs and IL-10

Serum titers of IgM and IgG anti-PDC-E2 autoantibodies were measured by ELISA using our well standardized recombinant PDC-E2 [37]. Serum IL-10 were measured by a commercial ELISA kit (Duoset; R&D Systems, Minneapolis, MN, USA)

### 2.5 Liver mononuclear cell quantitation and junctional assays

Liver tissues were harvested immediately after animals were sacrificed and perfused with PBS containing 0.2% BSA (PBS/0.2% BSA), passed through a 100 μm nylon mesh, and re-suspended in PBS/0.2% BSA. The parenchymal cells were removed as pellets after centrifugation at 50 g for 5 min and the non-parenchymal cells isolated using 40% and 70% Percoll (GE HealthCare Biosciences, Quebec, Canada). After centrifugation, collected cells were washed with PBS/0.2% BSA and viability of cells was confirmed by trypan blue dye exclusion. Before staining cells with a previously defined optimal dilution of monoclonal antibodies (Abs), the cells were pre-incubated with anti-CD16/32 (clone 93) to block non-specific FcRγ binding. Anti-CD3-PE-Cy7, Anti-CD3-APC, anti-CD4-APC-Cy7, anti-CD8a-PerCP-Cy5.5, anti-CD11b-PE, anti-CD19-FITC, anti-CD19-PE, anti-Ly6C-PE-Cy7, anti-Ly6G-PerCP-Cy5.5, anti-NK1.1-APC, anti-NK1.1-APC-Cy7 and anti-CD69-FITC Abs (Biolegend, San Diego, CA, USA) were used to stain for lymphocyte subsets and activation of liver mononuclear cells. For a lymphocyte functional assay, liver mononuclear cells were treated with 50 ng/ml phorbol-myristate acetate (PMA, Sigma Aldrich) and 1 mg/ml ionomysin (Sigma Aldrich) in the presence of brefeldin A (10 μg/ml) (BD Biosciences, San Diego, CA, USA) for 4 hours, followed by incubation with anti-CD3-FITC, anti-CD3-PE-Cy7 anti-CD4-APC-Cy7, anti-CD8a-PerCP-Cy5.5 and anti-NK1.1-APC, anti-FasL-PE, and anti-CD107a-APC-Cy7 Abs (Biolegend), permeabilized with Cytofix/Cytoperm reagent (BD Biosciences), and stained with anti-IFN-γ-PE-Cy7 and anti-granzyme B-FITC Abs. Stained cells were assessed on the BD FACSVerse (BD Biosciences) and data analyzed by FlowJo software (Tree Star, Inc., Ashland, OR, USA).

### 2.6 Isolation of mRNA and real-time PCR

Total RNA from liver specimens was obtained by the TRI$^{zQl}$ method (Invitrogen Life Technologies, Carlsbad, CA, USA). The cDNA was generated by oligonucleotide priming using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA, USA). Amplification was performed with SYBR Green MasterMix (Thermo Scientific, USA) using the 7500 Real-Time PCR System (Applied Biosystems). Results were analyzed by $2^{-\Delta\Delta Ct}$ relative quantification method and normalized to β-actin. Primer sequences used in PCR are as described previously [36].

### 2.7 Histopathology

Excised liver tissues were immediately fixed with 10% buffered formalin solution for 2 days at room temperature and thereafter embedded in paraffin. Paraffin-embedded tissues were then cut into 4-μm sections for routine hematoxylin and eosin (H-E) and 5-μm sections for Masson's trichrome staining.

### 2.8 Statistical analysis

Differences between two groups were evaluated using two-sided unpaired Student's t test. Comparison of more than two groups was performed with one-way ANOVA followed by Tukey multiple comparison test. Statistically significant differences were defined as p values of less than 0.05, less than 0.01, and less than 0.001.

## 3. Results

### 3.1 Increased autoimmune cholangitis and liver fibrosis in 2-OA-OVA immunized IL-10 knockout mice.

To define the role of endogenous IL-10 in the pathogenesis of autoimmune cholangitis, we first immunized IL-10 knockout mice and littermate wild types with 2-OA-OVA and monitored disease progress. At 10 weeks post-immunization, IL-$10^{-/-}$ mice immunized with 2-OA-OVA had a significant increase in portal inflammation and fibrosis (Fig. 1A). These histologic observations are supported by increase in liver lymphocytic infiltrates and liver collagen I and III expressions in 2-OA-OVA immunized IL-$10^{-/-}$ mice compared to 2-OA-OVA immunized wild type mice (Fig. 1B and 1C). Lymphocyte subsets including T, NK, and NKT cells were all significantly increased in 2-OA-OVA immunized IL-$10^{-/-}$ mice, while B cells were not different between groups (Fig. 1D). The absolute numbers of $CD4^+$ and $CD8^+$ T cells were also increased in 2-OA-OVA immunized IL-$10^{-/-}$ mice (Fig. 1E). In addition, anti-PDC-E2 IgG was significantly increased in 2-OA-OVA immunized IL-$10^{-/-}$ mice compared to 2-OA-OVA immunized littermates ($p<0.05$) (Fig. 1F). Taken together, mice deficient in IL-10 had more severe autoimmune cholangitis and fibrosis, suggesting that endogenous IL-10 is critical in the maintenance of immune tolerance in liver.

### 3.2 AAV-IL-10 administration 3 days before 2-OA-OVA immunization increased fibrosis and $CD4^+$ T cell activation.

To investigate whether administration of IL-10 could prevent autoimmune cholangitis, we administered a single intravenous injection of AAV-IL-10 or mock virus to female C57BL/6 mice 3 days before 2-OA-OVA immunization and studied at 10 weeks. As shown in Figure 2A, IL-10 was expressed in the serum of AAV-IL-10 ($3\times10^7$ TU/mouse) injected mice at a level around 850 pg/ml (Fig. 2A). Histologically, there were no differences in portal inflammation among the three groups (Fig. 2B, H&E stain). Consistent with these observations, there were no differences in the numbers of total liver lymphocytes (Fig. 2C). However, the numbers of T cells were increased but the numbers of B cells were decreased in AAV-IL-10 injected mice (Fig. 2D). The numbers of $CD4^+$ T cells were significantly increased in AAV-IL-10 injected mice while the numbers of $CD8^+$ T cells were similar among the three groups (Fig. 2D and 2E). The frequency of $CD69^+$ activated T cells was also increased in mice injected with AAV-IL-10 (Fig. 2F). To our surprise, liver IFN-γ production and collagen expression were significantly increased in 2-OA-OVA immunized mice injected with AAV-IL-10 (Figures 2G and 2H) concomitant with increased fibrosis (Fig. 2B, Trichrome stain).

To determine the dose effect of IL-10 in autoimmune cholangitis, we injected C57BL/6 mice with a lower dose ($1\times10^7$ TU/mouse) of AAV-IL-10 3 days before 2-OA-OVA immunization. Serum levels of IL-10 were approximately 150 pg/ml (Fig. 3A). There were no differences in the levels of liver lymphocyte infiltrates, expressions of liver IFN-γ and collagen I and III in the low dose AAV-IL-10 treated 2-OA-OVA immunized mice and control mice (Fig. 3).

Thence to further clarify the proinflammatory effect of AAV-IL-10 in autoimmune cholangitis, we evaluated the activation and functions of liver T, NK and NKT cells of high dose AAV-IL-10 treated mice. The frequency of activated $CD4^+$ T cells and levels of IFN-γ production were higher in AAV-IL-10 treated mice when compared with AAV-mock and normal saline treated mice (Fig. 4A). The expression levels of granzyme B, FasL, and CD107a (lysosome-associated membrane protein-1; LAMP-1), a marker of cytotoxic cell degranulation, were higher in liver $CD8^+$ T cells and NKT cells of AAV-IL-10 treated mice than AAV-mock and normal saline treated mice (Fig. 4B and 4C). In addition, the expression levels of granzyme B and FasL in liver NK cells were also higher in AAV-IL-10 treated mice than in AAV-mock and normal saline treated mice (Fig. 4D). Taken together, AAV-IL-10 administration increased autoimmune cholangitis by increasing activation and function of T, NK and NKT cells.

### 3.3 AAV-IL-10 administration 3 weeks after 2-OA-OVA immunization promoted portal inflammation.

To focus on why IL-10 alters the onset of disease, we administered AAV-IL-10 three weeks after mice were immunized with 2-OA-OVA. Portal inflammation was more severe in AAV-IL-10 treated mice than AAV-mock and normal saline treated mice. However, there was no difference in the degree of fibrosis or liver collagen I and III expression amongst the three groups (Fig. 5A and 5C). In AAV-IL-10 treated mice, the numbers of hepatic lymphocytes, hepatic T cells including $CD4^+$ and $CD8^+$ T cells, and the frequency of $CD69^+$ activated T cells were significantly increased when compared with AAV-mock and normal saline treated mice (Fig. 5B, 5D, 5E and 5F). In addition, liver IFN-γ expression was significantly increased in AAV-IL-10 treated mice ($p<0.001$) (Fig. 5G). Taken together, AAV-IL-10 treatment exacerbates autoimmune cholangitis even after disease onset.

### 3.4 AAV-IL-10 administration inducedfibrosis and lymphocyte activation in non-immunized mice.

We then injected AAV-IL-10 at a dose of $3\times10^7$ TU/mouse to naive mice and examined the liver 10 weeks later. Average serum levels of IL-10 were 870 pg/ml in AAV-IL-10 injected mice (Fig. 6A). As shown in Figure 6, there were no pathological damages in AAV-mock injected mice but cell infiltrations and collagen deposition noted in the livers of AAV-IL-10 injected mice (Fig. 6B). Furthermore, an increase in inflammatory monocytes ($Ly6G^-CD11b^+Ly6C^{hi}$) and inflammatory macrophages (Ly6G'$CDnb^+Ly6C^{lo}$) were evident in the livers of AAV-IL-10 administered mice when compared with AAV-mock control mice (Fig. 6C and 6D). The numbers of lymphocytes in the livers were also significantly increased in AAV-IL-10 administered mice (Fig. 6E). The levels of proinflammatory cytokines and chemokines IFN-γ, TNF-α, CXCL9 and CXCL10 were significantly elevated in AAV-IL-10 treated mice. In addition, collagen I and III were also elevated in AAV-IL-10 treated mice. However, there were no statistical difference in the levels of expressions of profibrotic cytokine IL-4 in the liver of AAV-IL-10 mice compared with AAV-mock controls (Figure 6F). As expected, AMA were not detected in AAV-IL-10 treated mice (data not shown).

### 3.5 Summary

The effects of IL-10 in autoimmune cholangitis and non-immunized mice were summarized in Table 1.

## 4 Discussion

IL-10 is predominantly an immunosuppressive and anti-inflammatory cytokine. Given the anti-inflammatory and anti-fibrotic properties of IL-10, we examined the therapeutic potential of IL-10 in our chemical induced murine model of autoimmune cholangitis. We used recombinant adeno-associated virus to express IL-10 in mice because recombinant IL-10 protein has only a short half-life and multiple treatments are needed [38]. Genetically engineered recombinant AAV is an appropriate vector for *in vivo* gene transfer because of its replication defective nature, capability of infecting a broad range of cell types including non-dividing cells, presence *in vivo* as concatemers for long-term expression and eliciting only a mild immune response compared with the older versions of adenovirus [39]. In this study, we applied AAV-DJ, a recombinant AAV produced by a complex library of hybrid capsids from 8 different wild-type viruses. AAV-DJ has a superior transduction efficiency in liver and is an attractive vector for liver-specific gene expression [4, 36, 40]. Our data demonstrated that IL-10 was highly expressed in the serum of AAV-IL-10 injected mice up to at least 10 weeks post injection. In contrast, mice injected with AAV-mock remained healthy without changes in body weight or abnormalities in liver histopathology.

Although the serum levels of IL-10 are higher in patients with PBC, the role of IL-10 is unclear [41]. Our data in this study reflect that autoimmune cholangitis and fibrosis are more severe in 2-OA-OVA immunized IL-10 deficient mice than in wild type mice, suggesting that endogenous IL-10 is necessary in the maintenance of immune tolerance. It is consistent with previous studies that mice deficient in IL-10 had increased hepatotoxicity and proliferative response of hepatocytes in the acute carbon tetrachloride ($CCl_4$) induced model of liver injury [28] and elevated transaminase activities in plasma and liver inflammation in ConA-induced liver damage [29, 42].

Mice treated with AAV-IL-10 before and after the onset of clinical disease resulted in increased liver inflammation and fibrosis. Recruitment and activation of IFN-γ expressing $CD4^+$ T cells and granzyme B, FasL, or CD107a expressing $CD8^+$ T, NK and NKT cells were increased in AAV-IL-10 administered PBC mice than in AAV-mock administered mice. In PBC, Th1 cells and IFN-γ highly contribute in the disease process by increasing immune cell infiltrates, upregulating MHC class II expression in antigen presenting cells, promoting anti-PDC-E2 antibodies production, as well as activating $CD4^+$ and $CD8^+$ T cells and NK cells [4, 43]. Activated NK cells, NKT cells and $CD8^+$ T cells express FasL or secrete cytotoxic granules such as perforin and granzyme and thus induce biliary destruction [5]. Hence, exogenous IL-10 can increase inflammation in AAV-IL-10 treated mice through recruiting and activating $CD4^+$ T, $CD8^+$ T, NK and NKT cells and increasing bile duct damage.

IL-10 is most recognized for its anti-inflammatory properties. However, IL-10 can also exhibit proinflammatory activities during some inflammatory responses [44], i.e.

endotoxemia [45], tissue transplantation [46], and in systemic lupus erythematosus (SLE) [47]. Large amounts of IL-10 have been detected in the ConA model of liver fibrosis in mice [48, 49]. In addition, in human *in vivo* studies, administration of recombinant human IL-10 to patients with Crohn's disease or psoriasis induces proinflammatory cytokine IFN-γ [50, 51]. Notably, the administration of recombinant human IL-10 induces antitumor immunity: infiltration and activation of intratumor tumor-specific cytotoxic $CD8^+$ T cells, expression of the IFN-γ and granzymes in $CD8^+$ T cells, and intratumor antigen presentation molecules [52, 53]. In this study, administration of AAV-IL-10 leads to increases in IFN-γ of $CD4^+$ T cells and granzyme B, FasL, and/or CD107a in $CD8^+$ T, NK and NKT cells. In addition, the inflammatory effect of IL-10 is evident in that mice treated with only IL-10 resulted in recruitment of lymphocytes and inflammatory monocytes/macrophages in liver.

Interestingly, administration of AAV-IL-10 increased collagen deposition both in our 2-OA-OVA mouse model of autoimmune cholangitis and naive mice, suggesting a potential role of IL-10 in liver fibrosis. In murine lung fibrosis, pulmonary overexpression of IL-10 by adenoviral gene transfer during silica-induced lung inflammation and fibrosis exacerbates fibrotic lesions by exacerbating the Th2 response as well as the production of the profibrotic cytokines IL-4 and IL-13 [54]. In another study, transgenic mice with lung specific long-term overexpression of IL-10 exhibites substantial cellular accumulation around airways and regions with lung fibrosis, which is mediated by recruiting fibrocytes and activation of M2 macrophages via the CCL2/CCR2 axis [55]. In this study, increased lymphocytes, monocytes/macrophages and inflammatory cytokines and chemokines in mice treated with AAV-IL-10 were noted whereas the profibrotic cytokine IL-4 was not increased.

Early work demonstrated that IL-10 is capable of enhancing activation and proliferation of various immune cell types, such as $CD4^+$ and $CD8^+$ T cells, NK cells, and B cells [53, 56–60]. IL-10 cellular responses require specific recognition and assembly of a heterodimeric cell surface complex comprised of the IL-10R1 and IL-10R2 chains. IL-10R1 expression is restricted mainly to immune cells and specific to IL-10, while IL-10R2 is a low affinity shared receptor that participates in receptor complexes with other IL-10 family members IL-19, IL-20, IL-22, IL-24, and IL-26 [61]. Upon engagement of IL-10R, the Janus kinase/Signal Transducer and Activator of Transcription (JAK/STAT) pathway is commonly used to regulate the transcription of IL-10-responsive genes. STAT3 is an essential protein downstream of IL-10 in this pathway, although interactions with other STAT proteins such as STAT1 may mediate cell- or tissue-specific roles [62]. Of note, STAT1 is typically activated by IFN-γ and mediates much of its proinflammatory activity [21]. TGF-β1 is also an immunosuppressive cytokines in down regulation of inflammation. However, it is a critical fibrotic cytokine which plays an important role in the initiation and progression of liver fibrosis [63]. In this study, administration of IL-10 increases IFN-γ production in $CD4^+$ T cells. IL-10 administration is therefore a too simplistic approach to accomplish immunosuppressive effect in autoimmune cholangitis.

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## Abbreviations:

| | |
|---|---|
| **2-OA-OVA** | 2-octynoic acid conjugated ovalbumin |
| **AAV** | adeno-associated virus |
| **AMAs** | anti-mitochondrial antibodies |
| **PBC** | primary biliary cholangitis |
| **TU** | transduction unit |

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## Highlights

- The endogenous IL-10 is critical in the maintenance of immune tolerance in liver.
- The exogenous administration of IL-10 exacerbates liver inflammation and fibrosis in autoimmune cholangitis.
- The exogenous IL-10 can increase inflammation in AAV-IL-10 treated mice through recruiting and activating $CD4^+$ T, $CD8^+$ T, NK and NKT cells.
- Any immune therapeutic regimen that impacts IL-10 may be a two edge sword, potentially have both benefits and harm.

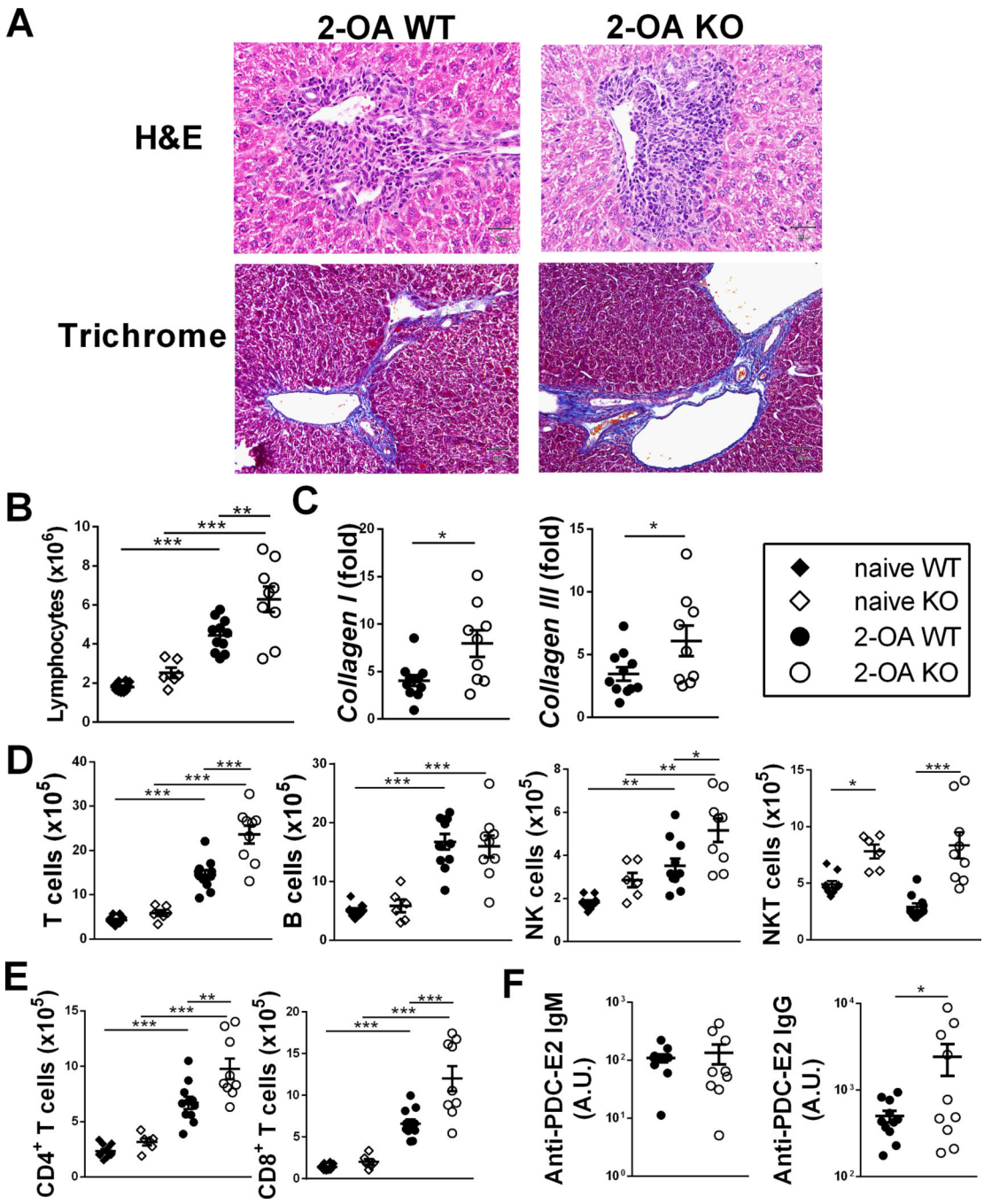


**Fig. 1. Increased AMA, collagen levels and cell infiltration and activation in 2-OA-OVA immunizedIL-10 knockout mice.**

IL-10 knockout mice and wild type littermates were immunized with 2-OA-OVA and sacrificed at 10 weeks post-immunization. (A) Representative H&E staining (x400 magnification) and Masson's trichrome staining (x200 magnification) of liver sections. The collagen fibers are stained blue. (B) Liver lymphocytes were quantified. (C) The expressions of collagen I and collagen III mRNA in the liver were detected by RT-qPCR. (D) The numbers of T, B, NK, and NKT cells in the liver were quantified. (E) The numbers of $CD4^+$ and $CD8^+$ T cells in the liver were quantified. (F) Serum levels of anti-PDC-E2 IgM and IgG

were determined by ELISA. A.U., arbitrary unit. WT, wild type; KO, knockout. Each dot represents an individual mouse. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

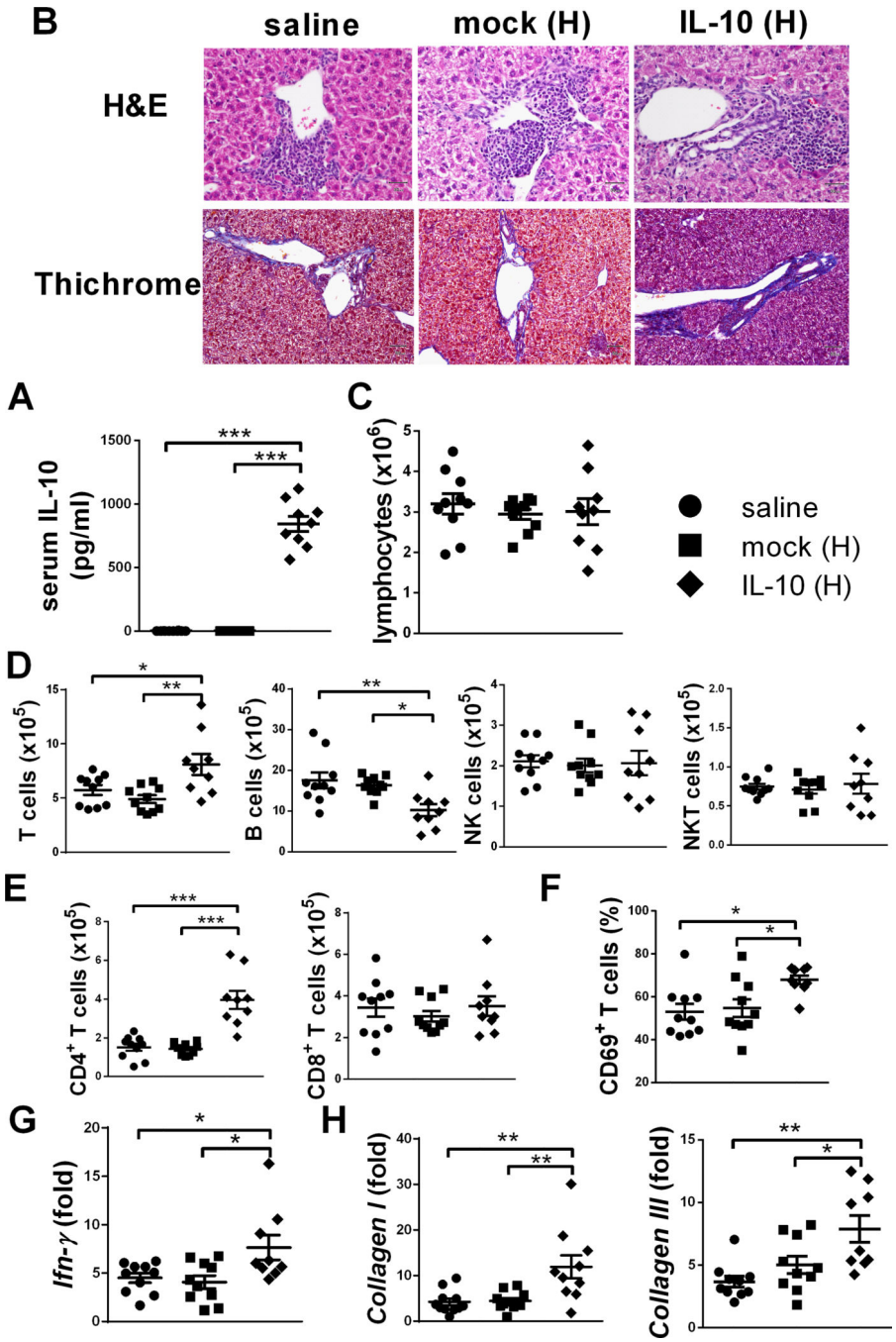


**Fig. 2. AAV-IL-10 treatment before 2-OA-OVA immunization increased liver inflammation and fibrosis.**

Female C57BL/6 mice were injected with AAV-IL-10, AAV mock ($3\times10^7$ TU/mouse) or normal saline three days before the first 2-OA-OVA immunization and sacrificed at week 10 after the first 2-OA-OVA immunization. (A) Serum IL-10 was determined by ELISA. (B) Representative H&E staining (x400 magnification) and Masson's trichrome staining (x200 magnification) of liver sections. The collagen fibers are stained blue. (C) Liver lymphocytes were quantifies. (D, E) The numbers of (D) T, B, NK and NKT cells and (E) $CD4^+$ and $CD8^+$ T cells in the liver were quantified. (F) The percentages of $CD69^+$ on T cells in the

liver were measured. (G) The expressions of IFN-γ mRNA in the liver were determined by RT-qPCR. (H) The expressions of collagen I and collagen III mRNA in the liver were determined by RT-qPCR. Each dot represents an individual mouse. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

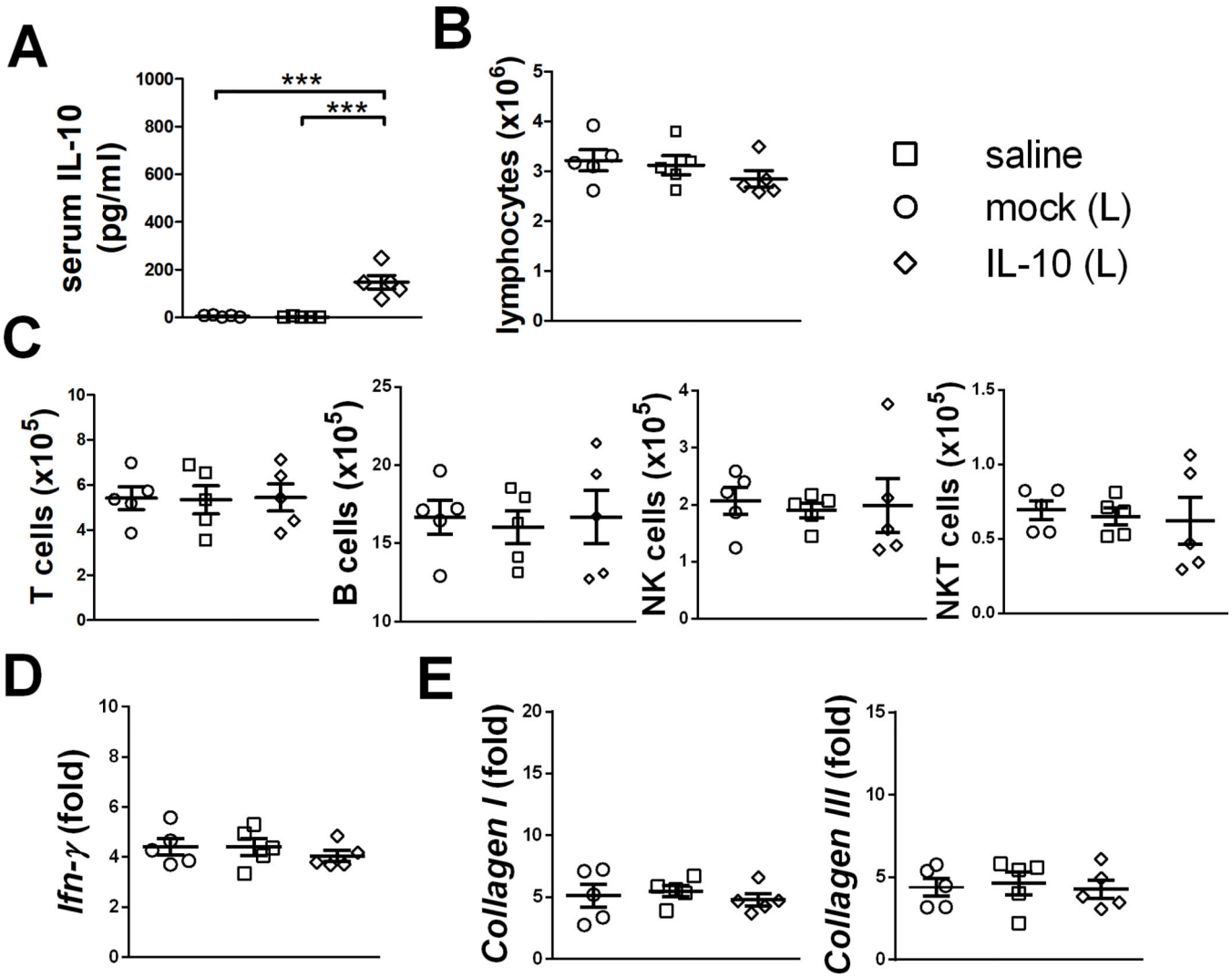


**Fig. 3. There was no effect in 2-OA-OVA immunized mice pretreated with low dose IL-10.**

Mice were injected with low doses of AAV-IL-10, AAV-mock ($1\times10^7$ TU/mouse) or normal saline three days before the first 2-OA-OVA immunization and sacrificed at 10 weeks later. (A) Serum IL-10 was measured by ELISA. (B) Liver lymphocytes were counted. (C) The numbers of T, B, NK and NKT cells in the liver were measured. (D) The expressions of IFN-γ mRNA in the liver were detected by RT-qPCR. (E) The expressions of collagen I and collagen III mRNA in the liver were detected by RT-qPCR. Each dot represents an individual mouse.

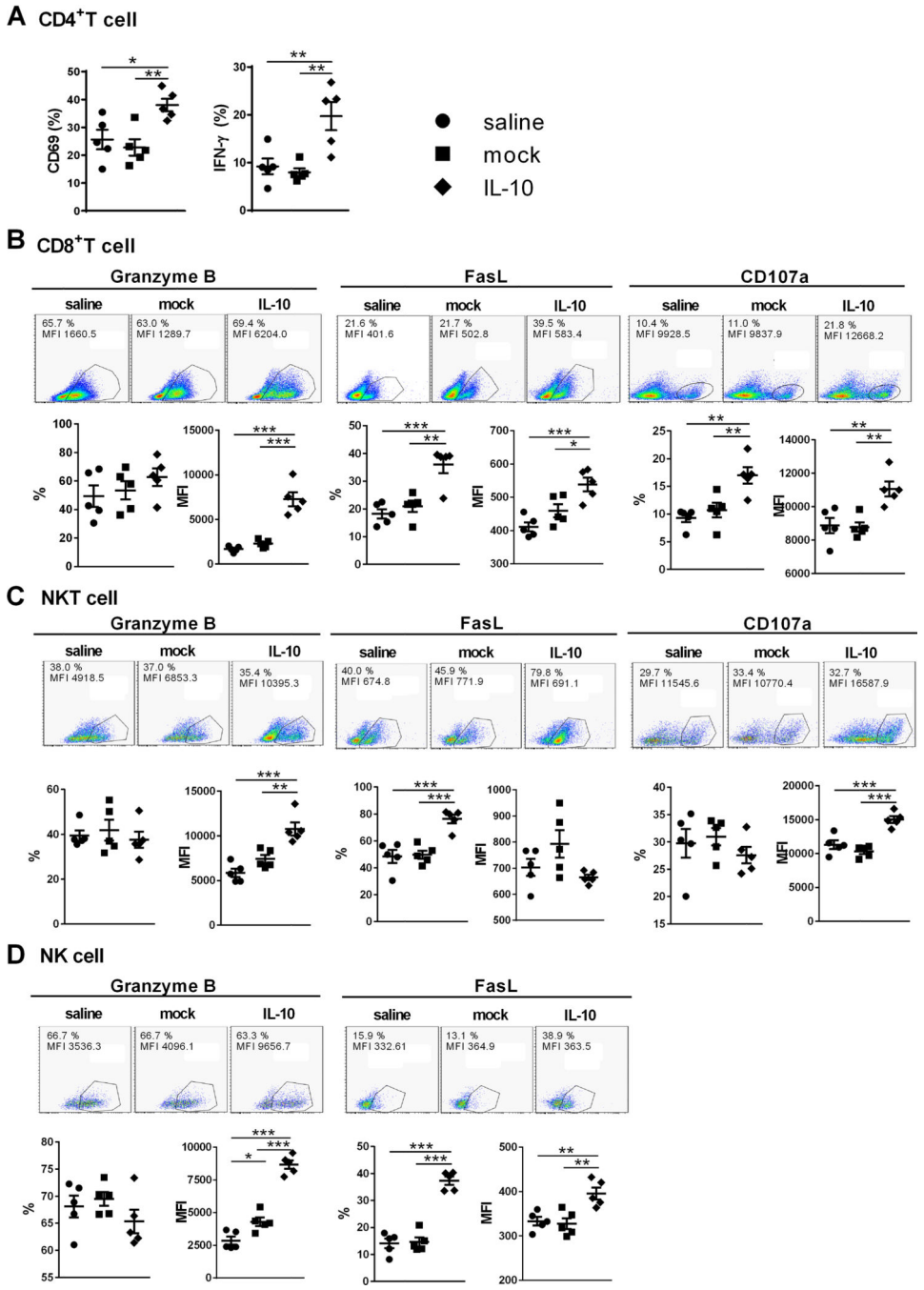


**Fig. 4. AAV-IL-10 treatment before 2-OA-OVA immunization increased hepatic immune cell function.**

C57BL/6 mice were injected with AAV-IL-10, AAV mock ($3\times10^7$ TU/mouse) or normal saline three days before the first 2-OA-OVA immunization. The activation and function of immune cells in the liver of mice were examined at week 5 post the first 2-OA-OVA immunization. (A) The frequency of $CD69^+$ and IFN-γ production in $CD4^+$ T cells were measured. (B) The percentages of granzyme B, FasL, and CD107a in $CD8^+$ T cells and their expression levels were measured by flowcytometry. (C) The percentages of granzyme B, FasL, and CD107a in NKT cells and their expression levels were measured by

flowcytometry. (D) The percentages of granzyme B and FasL in NK cells and their expression levels were measured by flowcytometry. MFI, mean fluorescence intensity. Each dot represents an individual mouse. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

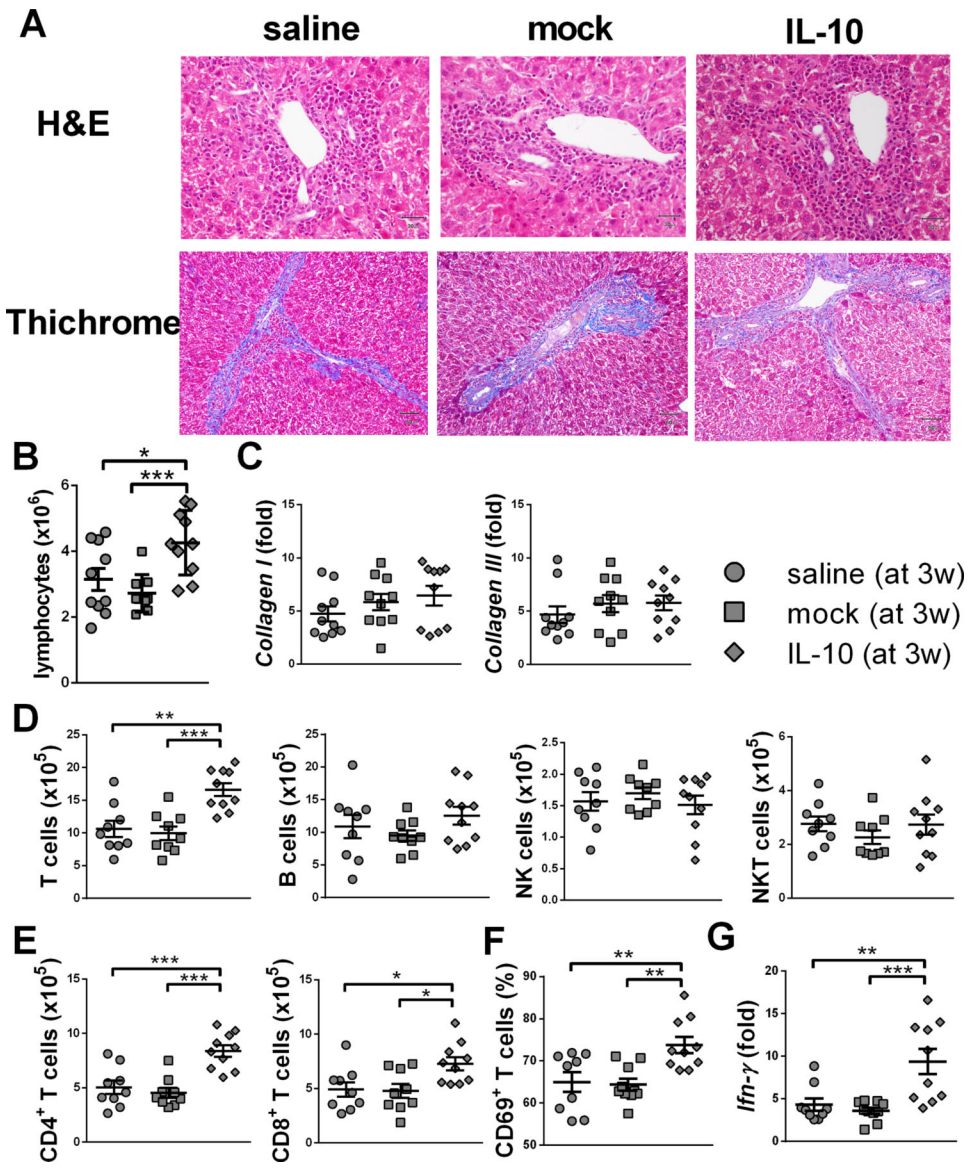


**Fig. 5. AAV-IL-10 delivery post 2-OA-OVA immunization also increased liver inflammation.**

Mice were injected with AAV-IL-10, AAV mock ($3\times10^7$ TU/mouse) or normal saline at three weeks after the first 2-OA-OVA immunization and sacrificed at week 10. (A) Representative H&E staining (x400 magnification) and Masson's trichrome stain (x200 magnification) of liver sections. The collagen fibers are stained blue. (B) Liver lymphocytes were quantified. (C) The expressions of collagen I and collagen III mRNA in the liver were determined by RT-qPCR. (D, E) The numbers of (D) T, B, NK and NKT cells and (E) $CD4^+$ and $CD8^+$ T cells in the liver were quantified. (F) The percentages of $CD69^+$ on T cells in the liver were measured. (G) The expressions of IFN-γ mRNA in the liver were determined by RT-qPCR. Each dot represents an individual mouse. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

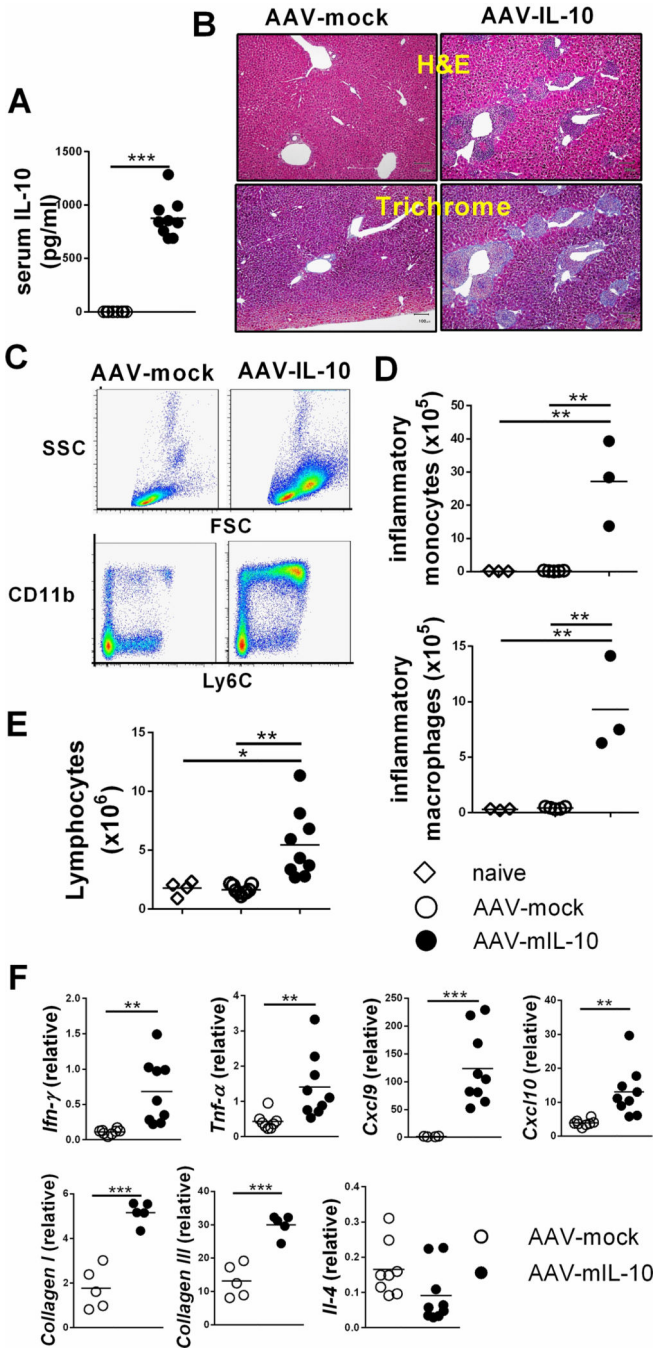


**Fig. 6. AAV-IL-10 treatment in naive mice resulted in liver inflammation andfibrosis.**

Naive mice were injected with AAV-IL-10 or AAV mock and sacrificed 10 weeks later. (A) Serum IL-10 was determined by ELISA. (B) Representative H&E staining and Masson's trichrome staining (x100 and magnification) of liver sections. The collagen fibers are stained blue. (C) Representative dot plots show forward scatter (FSC) /side scatter (SSC) and inflammatory monocytes (Ly6G$^-$ CD11b$^+$Ly6C$^{hi}$) and inflammatory macrophages (Ly6G$^-$ CD11b$^+$Ly6C$^{lo}$) of liver mononuclear cells of AAV mock and AAV-IL-10 administered mice. (D) Liver inflammatory monocytes and inflammatory macrophages were quantified.

(E) Liver lymphocytes were quantified. (F) The expressions of IFN-γ, TNF-α, CXCL9, CXCL-10, collagen I, collagen III and IL-4 mRNA in the liver were determined by RT-qPCR. Results presented as relative to internal β-actin expression (x1000). Each dot represents an individual mouse. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

**Table 1.**

Effects of IL-10 in liver.

| mice | PBC induction[#] | AAV-IL-10 | Features of liver inflammation[*] |
|---|---|---|---|
| IL-10$^{-/-}$ B6 | 2-OA-OVA immunization | None | Portal inflammation ↑<br>Fibrosis ↑<br>AMA IgG ↑ |
| B6 | 2-OA-OVA immunization | Three days before PBC induction<br>$3\times10^7$ TU/mouse | T cell actiovation ↑<br>Liver IFN-γ ↑<br>Fibrosis ↑ |
| B6 | 2-OA-OVA immunization | Three days before PBC induction<br>$3\times10^7$ TU/mouse | T cell actiovation ↑<br>Liver IFN-γ ↑<br>Fibrosis ↑ |
| B6 | 2-OA-OVA immunization | Three weeks after PBC induction<br>$1\times10^7$ TU/mouse | None |
| B6 | None | $3\times10^7$ TU/mouse | Liver inflammation ↑<br>Fibrosis ↑<br>Liver cytokines and chemokines ↑ |

[#]Mice with PBC induction had AMA, portal inflammation and fibrosis.

[*]Compare to control mice; ↑, increased