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Original Article

IGF2-NR4A2 Signaling Regulates Macrophage Subtypes to Attenuate Liver Cirrhosis

Lichao Yao[1#], Xue Hu[1#], Mengqin Yuan[1], Qiuling Zhang[1], Pingji Liu[1], Lian Yang[2], Kai Dai[1*] and Yingan Jiang[1*]

[1]Department of Infectious Diseases, Renmin Hospital of Wuhan University, Wuhan, Hubei, China; [2]Department of Obstetrics and Gynecology, Renmin Hospital of Wuhan University, Wuhan, Hubei, China

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Abstract

Background and Aims: Liver cirrhosis can lead to liver failure and eventually death. Macrophages are the main contributors to cirrhosis and have a bidirectional role in regulating matrix deposition and degradation. Macrophage-based cell therapy has been developed as an alternative to liver transplantation. However, there is insufficient evidence regarding its safety and efficacy. In this study, we aimed to explore the effect of combining insulin-like growth factor 2 (IGF2) with bone marrow-derived macrophages (BMDMs) to treat mice with liver cirrhosis. ***Methods:*** We assessed liver inflammation, fibrosis regression, liver function, and liver regeneration in mice with CCl_4-induced cirrhosis and treated with BMDM only or IGF2 + BMDM. We performed *in vitro* experiments in which activated hepatic stellate cells (HSCs) were co-cultured with macrophages in the presence or absence of IGF2. The polarity of macrophages and the degree of inhibition of HSCs were examined. The effect of IGF2 on macrophages was also verified by the overexpression of IGF2. ***Results:*** Combining IGF2 with BMDM reduced liver inflammation and fibrosis and increased hepatocyte proliferation. Combining IGF2 with BMDM was more effective than using BMDM alone. *In vitro* experiments demonstrated that IGF2 inhibited HSCs activation by upregulating NR4A2 to promote the anti-inflammatory macrophages phenotype. IGF2 also increased the synthesis of matrix metalloproteinases (MMPs) by macrophages, which may explain why administering IGF2 combined with BMDM was more effective than administering BMDM only. ***Conclusions:*** Our study provides a theoretical basis for the future use of BMDM-based cell therapy to treat liver cirrhosis.

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Introduction

Liver fibrosis is an excessive wound healing response to persistent liver injury in chronic liver diseases. In the advanced stages of the disease, the excessive deposition of extracellular matrix (ECM) causes cirrhosis, leading to portal hypertension and liver failure.[1] The development of liver cirrhosis critically depends on the inflammatory stage, leading to the infiltration of immune cells, especially macrophages, and an increase in stromal-expressing cell types. Moreover, liver-resident macrophages (Kupffer cells) are activated and release transforming growth factor-β (TGF-β) and other proinflammatory cytokines that activate hepatic stellate cells (HSCs).[2,3] Activated HSCs are the main cell population responsible for producing ECM proteins and profibrotic cytokines that have a pivotal role in the pathogenesis of cirrhosis.[4] Currently, no effective antifibrosis therapies are available. Alternative therapies for cirrhosis that are under development include antiviral, HSCs-targeted, and cell therapies, which have been tested in various cell populations, including bone marrow cells, mesenchymal stem cells, hepatocytes, and macrophages.[5–7] However, they have shown limited and inconsistent efficacy because of the incomplete understanding of the cellular and molecular mechanisms.

Inflammatory monocytes, are macrophage precursors, and tissue-resident macrophages are crucial regulators of tissue repair, regeneration, and fibrosis. Macrophages are highly plastic cells that can change their phenotype in response to external stimuli. Classically activated macrophages (M1) produce various proinflammatory mediators that recruit and activate other immune cells. Alternatively activated macrophages (M2) participate in anti-inflammatory responses, where the immune response is suppressed, and tissue regeneration and wound healing are promoted.[8] As liver fibrosis progresses, injury-induced inflammation recruits bone marrow-derived macrophages (BMDMs) to the liver, where they produce cytokines and chemokines that activate HSCs.[9,10] When liver fibrosis regresses, macrophages stop producing fibrotic cytokines and inflammatory factors and secrete matrix metalloproteinases (MMPs), which degrade the ECM.[1] Macrophage transplantation was tested as a cell

Keywords: Liver cirrhosis; Macrophage; IGF2; Cell therapy; Hepatic stellate cell.
Abbreviations: BMDM, bone marrow-derived macrophage; ECM, extracellular matrix; HSC, hepatic stellate cell; IGF, insulin-like growth factor; MMP, matrix metalloproteinase; NR4A, nuclear receptor family 4A.
[#]Contributed equally to this work.
[*]**Correspondence to:** Kai Dai and Yingan Jiang, Department of Infectious Diseases, Renmin Hospital of Wuhan University, #9 Zhangzhidong Road, Wuhan, Hubei 430060, China. ORCID: https://orcid.org/0000-0001-7270-9965 (KD) and https://orcid.org/0000-0003-4912-7494 (YJ). Tel/Fax: +86-13135656945 (KD) and +86-13720389866 (YJ), E-mail: daikai@whu.edu.cn (KD) and jiangya_cn@aliyun.com (YJ)

therapy in experimental fibrosis models. Interleukin (IL)-10/TGF-β-modified M2 macrophages significantly reduced renal inflammation and structural damage in mice with Adriamycin-induced nephropathy.[11] Thomas *et al*.[12] reported that BMDM infusion effectively treated liver fibrosis in mice.

The insulin-like growth factor (IGF) system comprises IGF1 and IGF2, specific receptors, binding proteins (IGFBPs 1–6), and IGFBP-specific proteases, which contribute to physiological functions such as organ development, growth, and regeneration. The components of the IGF system are hepatoprotective and significantly contribute to the regulation of hormones and metabolism.[13] Previous studies have shown that IGF2 was negatively correlated with inflammation and fibrosis in liver lobules, and that the concentration of IGF2 in the circulation was reduced in patients with liver cirrhosis and is significantly associated with liver dysfunction.[14,15] Giraudi *et al*.[16] confirmed that the IGF2 level significantly decreased as liver fibrosis progressed and proposed IGF2 as a biomarker to diagnose moderate-to-severe liver fibrosis. However, how IGF2 regulates the hepatic inflammatory and fibrotic programs remains largely unknown.

Moreover, IGF2 is an important immunomodulatory mediator that confers anti-inflammatory properties to mature macrophages.[17] Recent studies have shown that IGF2 alleviated experimental autoimmune encephalomyelitis (EAE) and dextran sodium sulfate (DSS)-induced colitis by programming macrophages to acquire an anti-inflammatory phenotype.[18,19] Therefore, we hypothesize that IGF2 alleviates cirrhosis by suppressing liver inflammation through the previously mentioned mechanism.

Nuclear receptor family 4A (NR4A) is an important regulator of inflammation and immune homeostasis and consists of three members, NR4A1 (Nur77), NR4A2 (Nurr1), and NR4A3 (Nor-1), which are early response modulators to control inflammatory responses and promote their regression.[20] Bonta *et al*.[21] demonstrated that the ectopic expression of NR4A in human macrophages reduced the synthesis of proinflammatory cytokines including IL6, IL1β, and IL8 and chemokines including macrophage inflammatory protein 1 alpha (MIP1α) and monocyte chemoattractant protein-1 (MCP1). Toll-like receptor signaling significantly upregulates glucocorticoid receptors and NR4A2 in macrophages, which contribute to the negative feedback regulation of the inflammatory response.[22] Chen *et al*.[23] found that the expression of NR4A2 was decreased in liver fibrotic tissues and TGF-β-activated hepatocytes, and NR4A2 inhibited HSCs proliferation and reduced ECM accumulation through the MAPK pathway. Although NR4A receptors have an important regulatory role in inflammatory responses, the role of NR4A in the immune activity of liver macrophages during the development of cirrhosis is still poorly understood.

In this study, IGF2-NR4A2 was identified as a key checkpoint that regulated macrophage phenotypes and affected the progression of liver cirrhosis. We found that IGF2 promoted the anti-inflammatory macrophage phenotype and inhibited HSCs activation through the upregulation of NR4A2, thus slowing the progression of liver cirrhosis. Our study provides primary evidence supporting the protective effect of IGF2 combined with BMDM in liver cirrhosis and is a theoretical basis for future use of BMDM-based cell therapy to treat liver cirrhosis.

Methods

Animal experiments

Male C57BL/6 mice at 8 weeks of age were purchased from the Institute of Laboratory Animal Science, Chinese Academy of Medical Science (Beijing, China). After acclimation for 1 week, they were intraperitoneally injected with 10% CCl_4 (1.0 mL/kg body weight, dissolved in corn oil at a ratio of 1:9) twice a week for 8 weeks. Another group of mice was injected with an equal amount of corn oil as a control. After CCl_4 treatment for 6 weeks, the model mice were randomly divided into three groups and treated by tail-vein injection with (1) 1×10^6 BMDMs, in 100 μL phosphate buffered saline (PBS); (2) 1×10^6 BMDM pretreated with recombinant human IGF2 (PeproTech, Cranbury, NJ, USA) followed immediately by intraperitoneal injection with 50 ng IGF2; or (3) an equivalent amount of PBS. All groups were treated once a week for 2 weeks. Mice were sacrificed 48 h after the final CCl_4 injection. Serum and liver samples were collected for subsequent experiments. The Animal Care and Use Committee of Renmin Hospital of Wuhan University approved the animal experiments (No. 20211204).

Preparation of BMDMs

To obtain BMDMs, femur and tibia bone marrow from 6- to 9-week old mice were extracted as previously described,[24] the resulting bone marrow cells were resuspended in DMEM/F12-10 medium containing 10% FBS and 20 ng/mL macrophage colony-stimulating factor (M-CSF). The cells were cultured at 37°C with 5% CO_2 for 7 days to get mature primary macrophages. Fresh differentiation medium was substituted on days 3 and 5. IGF-2 (5 ng/mL) was added to some differentiation medium on days 1, 3, and 5.

Flow cytometry

The fluorochrome conjugated monoclonal antibodies used for flow cytometry were anti-F4/80-PerCP, anti-CD11b-FITC, anti-CD86-PE, and anti-CD206-APC, all antibodies were purchased from BD Bioscience (San Diego, CA, USA). After 30 min of incubation at 4°C in the dark, CD16/32 antibody added to block surface Fc receptors, and unbound antibodies were washed away with FACS wash buffer. After washing, cells were incubated with fixation/permeabilization buffer for 20 min at 4 °C. Flow cytometry was performed on a FACSCalibur flow cytometer using the CellQuest acquisition software (BD Biosciences, San Diego, CA, USA). Flow cytometry data were analyzed with FlowJo software.

Cell culture and treatment

The THP-1 and LX-2 cell lines were obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). THP-1 cells were maintained in RPMI-1640 medium (HyClone, Logan, UT, USA), and LX-2 cells were maintained in DMEM medium (HyClone) supplemented with 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and 100 U/mL penicillin-streptomycin (Beyotime, Shanghai, China) at 37°C with 5% CO_2. The medium was changed every 2–3 days. Human monocytic THP-1 cells were activated to nonpolarized M0 macrophages using 100 ng/mL PMA (MedChemExpress, Monmouth Junction, NJ, USA) for 24 h, polarized M1 macrophages using 10 ng/mL lipopolysaccharide (LPS) and 20 ng/mL IFN-γ for 24 h, and polarized M2 macrophages using 20 ng/mL IL4 and 20 ng/mL IL13 for 24 h. LPS, IFN-γ, IL4 and IL13 were purchased from Beyotime.

M0 macrophages were treated with a series of concentrations of recombinant human IGF2 (5, 10, 20, 50, and 100 ng/mL) at 30 m, 3 h, 6 h, 12 h, and 24 h. LX-2 cells were activated by 5 ng/mL TGF-β1 (PeproTech) for 24 h and then co-cultured with macrophages prepared from THP-1 cells in

Table 1. Primer sequences used for qRT-PCR

Gene	Forward sequence (5′-3′)	Reverse sequence (5′-3′)
Mouse		
Il6	GCCTTCTTGGGACTGATGCT	TGTGACTCCAGCTTATCTCTTGG
Il10	GCTCCAAGACCAAGGTGTCT	AGGACACCATAGCAAAGGGC
Tnf	TAGCCCACGTCGTAGCAAAC	ACCCTGAGCCATAATCCCCT
Il1b	TGCCACCTTTTGACAGTGATG	TTCTTGTGACCCTGAGCGAC
GAPDH	GCAGTGCCAGGTGAAAATCG	TACGGCCAAATCCGTTCACA
Human		
NR4A2	GCTGGACTCCCCATTGCTTT	GCCACGTAGTTCTGGTGGAA
CD80	AGGGGAAATGTCGCCTCTC	GTCCGGTTCTTGTACTCGGG
CD86	CAGGGACTAGCACAGACACAC	CAGGTTGACTGAAGTTAGCAGAG
CD163	GCGGGAGAGTGGAAGTGAAAG	GTTACAAATCACAGAGACCGCT
CD206	GGGAAAGGTTACCCTGGTGG	GTCAAGGAAGGGTCGGATCG
GAPDH	GAGAAGGCTGGGGCTCATTT	TAAGCAGTTGGTGGTGCAGG

two chambers separated by a semipermeable membrane with a pore size of 0.4 μm to permit the free exchange of secreted proteins between cell types. The cells were collected and analyzed after co-culture for 48 h.

Lentivirus transfection

Genechem Co. Ltd. (Shanghai, China) constructed a lentiviral vector overexpressing the target gene IGF2 (OE-IGF2) and a negative control (LV-NC). THP-1 cells were seeded in 6-well plates (5×10^5 cells per well) and cultured for 24 h before transfection. Lentiviral vectors were transfected into the cells according to the manufacturer's instructions. After transfection for 6 h, the culture medium was replaced with fresh medium for an additional 48 h incubation. Puromycin (2 μg/mL) was used to screen the cells infected with the virus carrying a puromycin resistance gene, and the stably transfected cells were selected for subsequent experiments. The transfection efficiency was determined by western blotting.

Histopathology

Liver tissue samples obtained from mice were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin (H&E) for general histopathological profiles and with Masson for collagen fiber. ImageJ software was used to analyze the Masson-positive areas. The percentage of the fibrotic area was calculated in five randomly selected fields per slide.

Immunohistochemistry

The 5 μm paraffin sections were dewaxed and rehydrated. A microwave oven was used for antigen retrieval for 5 m. To quench endogenous peroxidase activity, the sections were immersed in 0.3% H_2O_2 in PBS for 15 min. Subsequently, the sections were blocked for 30 m with 5% bovine serum albumin and incubated overnight with primary antibodies, anti-Ki67 (ab16667, 1:200; Abcam, Cambridge, UK) and anti-PCNA (ab92552, 1:200; Abcam) at 4 °C. After the sections were washed and incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies at room temperature, they were incubated for 2 m with 3,3′-diaminobenzidine solution and counterstained with hematoxylin. Image quantification was performed using ImageJ software.

Immunofluorescence staining

Liver sections were blocked using a solution containing 4% normal goat serum for 30 m at room temperature, permeabilized by 0.5% Triton X-100, and incubated with anti-Collangen1 (ab138492, 1:100; Abcam), and anti-α-SMA (A17910, 1:100; ABclonal, Wuhan, China) primary antibodies overnight at 4°C. Subsequently, they were incubated with Alexa Fluor 594-labeled anti-rabbit secondary antibody at room temperature for 1 h. Nuclei were stained with DAPI and observed under a fluorescence microscope.

Biochemical parameters

Mouse liver function was evaluated by serum levels of aspartate transaminase (AST), alanine transaminase (ALT), and alkaline phosphatase (ALP) were determined by standard methods with a Chemray 240 automatic biochemistry analyzer (Rayto, Shenzhen, China).

Real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using Trizol (Beyotime). NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) was used to determine the purity and concentration of RNA. Sangon Biotech Co. Ltd. (Shanghai, China) synthetized cDNA using BeyoRT reagent kits (Beyotime). PCR primers (Table 1) were synthesized by Sangon Biotech Co. Ltd. (Shanghai, China). GAPDH was used as an internal control. The reactions were performed in a Bio-Rad real-time PCR detection system using a SYBR Green qPCR Mix (Beyotime). After a pre-denaturation step at 95 °C for 5 min, 40 cycles of PCR were performed as follows: 10 s denaturation at 95 °C and 30 s annealing at 60 °C. The fold amplification for each gene was calculated using the $2-\Delta\Delta Ct$ method. Primer sequences are listed in Table 1.

Western blotting

Total protein was extracted by the RIPA lysis buffer and the protein concentration was determined with BCA protein assay kits (Beyotime). Approximately 20 μg of protein from each sample was separated by polyacrylamide gel electrophoresis for 2 h and transferred to a polyvinylidene fluoride membrane. The membrane was then blocked with 5% skim milk in Tris-buffered saline with Tween-20 for 1 h. The membranes were incubated with primary antibodies overnight

at 4°C: Collagen 1 (ab138492, 1:1,000; Abcam), α-SMA (A17910, 1:1,000; ABclonal), NR4A2 (ab54366, 1:1,000; Abcam), ERK1/2 (A4782, 1:10,000; ABclonal), p-ERK1/2 (AP0234, 1:1,000; ABclonal), IGF2 (ab262713, 1:1,000; Abcam), MMP13 (ab39012, 1:3,000; Abcam), Ki67 (ab16667, 1:1,000; Abcam), TGF-β1 (ab215715, 1:1,000; Abcam), MMP12 (A3713, 1:1,000; ABclonal), MMP2 (A6247, 1:1,000; ABclonal), and GAPDH (ab9485, 1:2,500; Abcam). Subsequently, the membrane was incubated with HRP-conjugated goat anti-rabbit secondary antibodies at room temperature for 1 h. Protein bands were visualized using ECL reagents and the intensity of each band was quantified using Image Lab software (version 4.1; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis

Data were analyzed with GraphPad Prism 6.0 and reported as means ± SEM. Differences among multiple comparisons were assessed by two-way analysis of variance. Between-group differences in normally distributed data were compared with two-tailed Student's *t*-tests. Correlations were tested by the Spearman method. *P*-values <0.05 were considered statistically significant.

Results

Cell treatment by IGF2 combined with BMDM alleviates liver cirrhosis in mice

Flow cytometry showed that 69.0% of the BMDMs obtained by *in vitro* differentiation induced by macrophage colony-stimulating factor (M-CSF) expressed macrophage markers F4/80 and CD11b (Fig. 1B). H&E and Masson staining showed that the hepatic lobule structures were unclear, hepatic sinusoids were disordered, more degenerative necrotic and immune cells were present, and the fibrosis areas were enhanced in mice with CCl_4-damaged livers. After BMDM treatment, both the infiltrated inflammatory cells and fibrotic areas in the liver tissues were significantly reduced, and this improvement was further enhanced in the BMDM + IGF2 group (Fig. 1C, D). We next measured the expression levels of fibrosis-related genes in the liver tissues, including TGF-β1, α-SMA, and collagen-1, all of which had lower protein expression levels in control mice. However, they significantly increased after CCl_4 administration. Notably, the expression levels of the genes were significantly reduced after BMDM treatment and were more reduced after treatment with IGF2 + BMDM (Fig. 1E–H). Similar results were obtained in the liver tissue immunofluorescence experiment. Compared with the CCl_4 group, the expression levels of α-SMA and Collagen-1 in both the BMDM and IGF2 + BMDM groups were significantly reduced, with the IGF2 + BMDM group showing lower expression levels (Fig. 2A–C). These results show that combining IGF2 with BMDM has a better therapeutic effect on liver cirrhosis.

Combining IGF2 with BMDM improves liver function and reduces inflammation in cirrhotic mice

We have examined the effects of combining IGF2 with BMDM on liver function and liver inflammation in cirrhotic mice. Biochemical measurement results showed that, compared with the control group, serum AST, ALT, and ALP levels in the CCl_4 group were significantly increased. These levels were decreased in both the BMDM and IGF2 + BMDM groups, with the decrease was more pronounced in the IGF2 + BMDM group (Fig. 2D–F). To examine the inflammatory state of the liver, we performed a qRT-PCR assay to examine the mRNA expression levels of *Il1b*, *Tnf*, *Il6*, and *Il10* in mouse liver tissue. The results showed that compared with the control group, the CCl_4 group showed significantly increased mRNA expression levels of inflammatory factors *Il1b*, *Tnf*, and *Il6*, but the expression of anti-inflammatory *Il10* showed no significant difference. Compared with the CCl_4 group, both the BMDM and the IGF2 + BMDM groups showed decreased mRNA expression levels of the previously mentioned inflammatory factors, while the expression of *Il10* was increased. Such changes were more pronounced in the IGF2 + BMDM group (Fig. 2G–J). These results show that administering IGF2 combined with BMDM was more effective in improving liver function and reducing liver inflammation than administering BMDM alone.

Combining IGF2 with BMDM promotes hepatocyte regeneration in cirrhotic mice

Hepatocyte proliferation is an important regenerative response observed in chronic liver injury. To determine whether hepatocyte proliferation was related to the improvement observed in liver cirrhosis after administering IGF2 combined with BMDM, we used immunohistochemistry to quantify Ki67-positive and PCNA-positive hepatocytes in the liver of mice. The results showed that few Ki67- and PCNA-positive cells were observed in both the control and model groups. However, the number of positive cells was significantly increased in the BMDM and IGF2 + BMDM groups, with the latter having the highest positive rate (Fig. 3A–C). The results of western blotting revealed a significant increase in the expression of Ki67 protein in the liver tissues of the IGF2+BMDM group compared with the CCl_4 group (Fig. 1E, K), indicating that combining IGF2 with BMDM can better stimulate hepatocyte regeneration in cirrhotic mice.

IGF2-NR4A2 pathway mediates macrophage polarization toward an anti-inflammatory phenotype

M0 macrophages were treated with a series of different concentrations of IGF2. qRT-PCR showed that different concentrations and intervention times of IGF2 significantly affected the NR4A2 mRNA expression levels. Within a certain range, the increase in the expression of NR4A2 depended on the concentration and intervention time of IGF2, but beyond that range, the expression of NR4A2 decreased (Fig. 4A–E). Therefore, based on the results of the qRT-PCR assay, subsequent experiments were performed using 10 ng/mL IGF2 and an intervention time of 12h. M1 and M2 macrophage markers revealed some changes in the macrophage subtypes. $CD80^+CD86^+$ cells represented the activated M1 macrophages, while $CD163^+CD206^+$ cells represented the activated M2 macrophages. The results of qRT-PCR assay showed that, after treatment with IGF2, M1 markers CD80 and CD86 were poorly expressed, while M2 markers CD163 and CD206 were significantly increased (Fig. 4F). Flow cytometry also demonstrated that, after treating M0 macrophages with IGF2, no $CD86^+$ cells were observed (Fig. 4G), while the number of $CD206^+$ cells accounted for 67.1% (Fig. 4H). The findings indicate that IGF2 promoted macrophage polarization toward the M2 anti-inflammatory phenotype. To explore the possible mechanism by which IGF2 promoted M2 polarization, we performed western blot assay to examine the expression of p-ERK1/2 in macrophages. The results showed that IGF2 activated the ERK1/2 pathway and promote the expression of NR4A2. Furthermore, when the ERK inhibitor FR180204 was added, the expression of p-ERK1/2 was inhibited, and NR4A2 expression was significantly reduced (Fig. 4I–K). More important, we found that, in the control group, the expression of NR4A2 was observed mainly in the cytoplasm. After IGF2 treatment, the expression of NR4A2 was increased mainly in

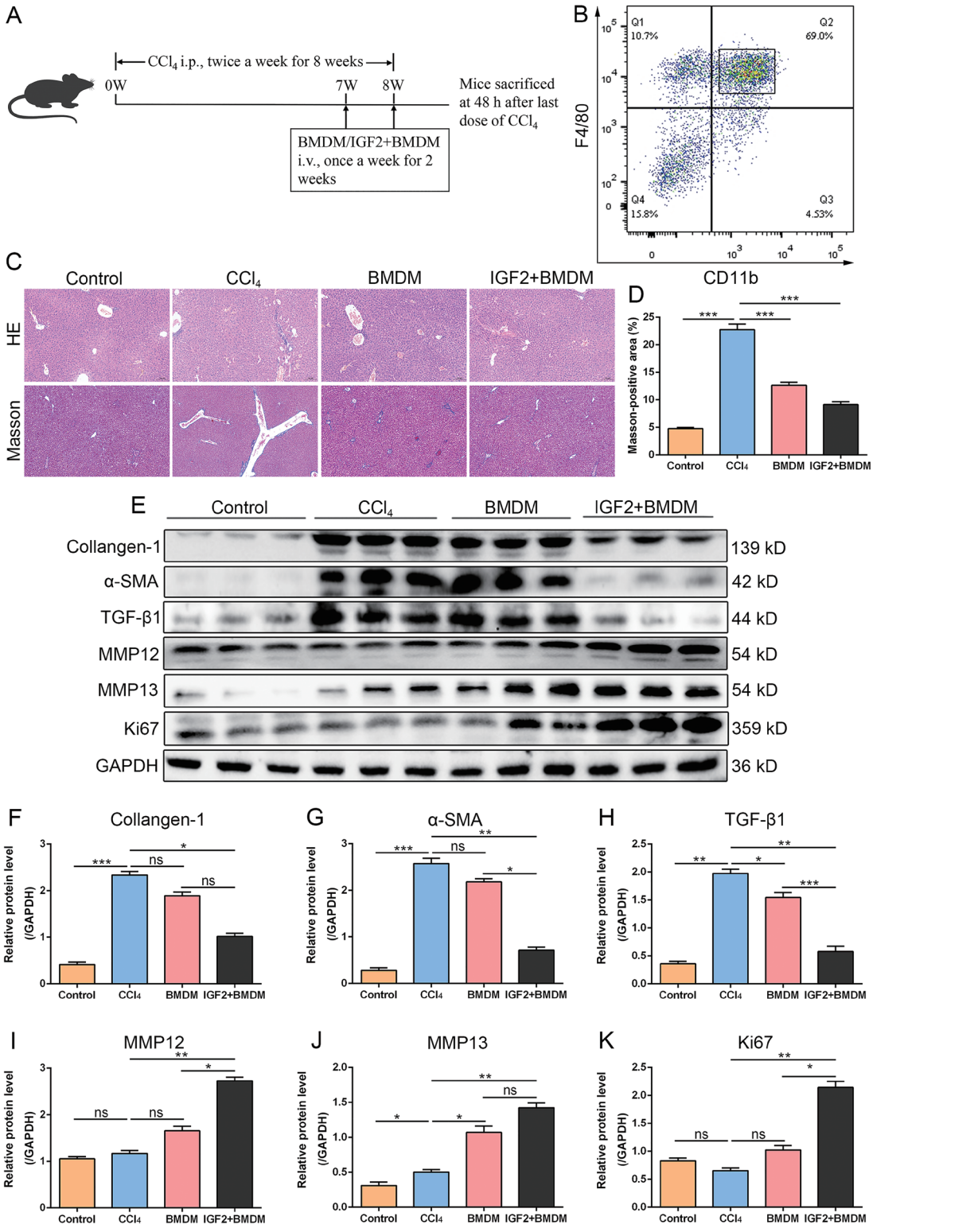

Fig. 1. Cell therapy of IGF2 combined with BMDM alleviates liver cirrhosis in mice. (A) Schematic represents the experimental design for inducing liver cirrhosis in mice, cell therapy and analysis in this study. (B) *In vitro* induced mouse BMDM (F4/80$^+$ CD11b$^+$ cells) was analyzed by flow cytometry. (C) Representative H&E and Masson staining of mice in each group, scale: 100 μm. (D) Quantification of Masson staining positive region. (E–K) Protein expression of Collagen-1, α-SMA, TGF-β1, MMP12, MMP13, and Ki67 in liver tissues was assayed by western blotting. Data are means ± SEM. *p<0.05, **p<0.01, ***p<0.001; ns, not significant. BMDM, bone marrow-derived macrophages; IGF2, insulin-like growth factor 2; α-SMA, α-smooth muscle actin; TGF-β1, transforming growth factor beta 1; MMP12, matrix metalloproteinase 12; MMP13, matrix metalloproteinase 13.

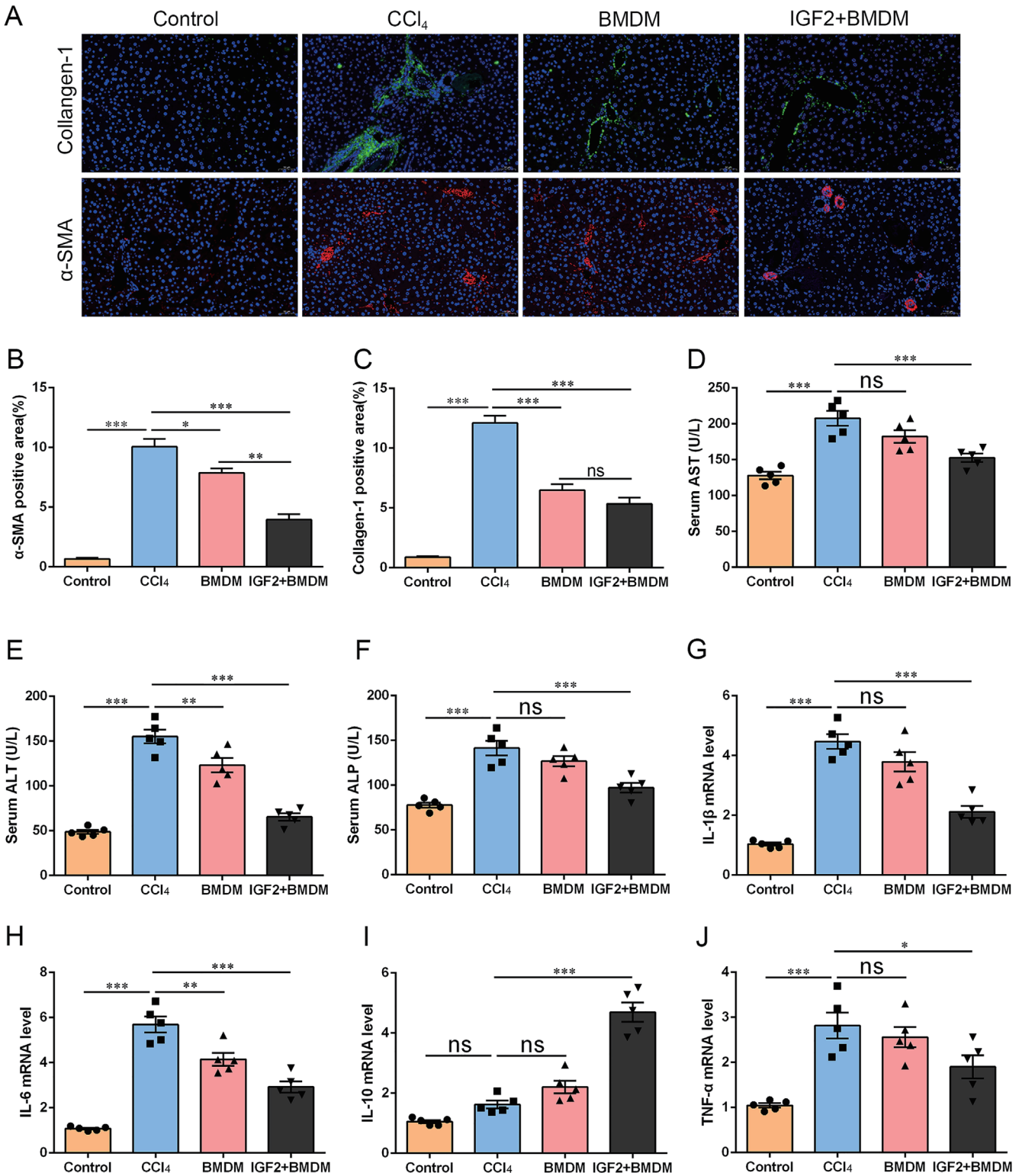

Fig. 2. Combining IGF2 with BMDM improves liver function and reduces inflammation in cirrhotic mice. (A) Representative images of collagen-1 (green) and α-SMA (red) staining in mouse liver, scale: 50 μm. Quantification of (B) α-SMA and (C) collagen-1 staining. (D–F) Biochemical analysis of liver function indicators AST, ALT, and ALP in the serum of mice from all groups. (G–J) mRNA expression of inflammation-related factors Il1b, Tnf, Il6, and Il10 in liver tissue from mice from each group. Data are means ± SEM (n=5 mice in each group). *p<0.05, **p<0.01, ***p<0.001; ns, not significant. BMDM, bone marrow-derived macrophages; IGF2, insulin-like growth factor 2; α-SMA, α-smooth muscle actin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; Il1b, interleukin 1b; Tnf, tumor necrosis factor; Il6, interleukin 6; Il10, interleukin 10.

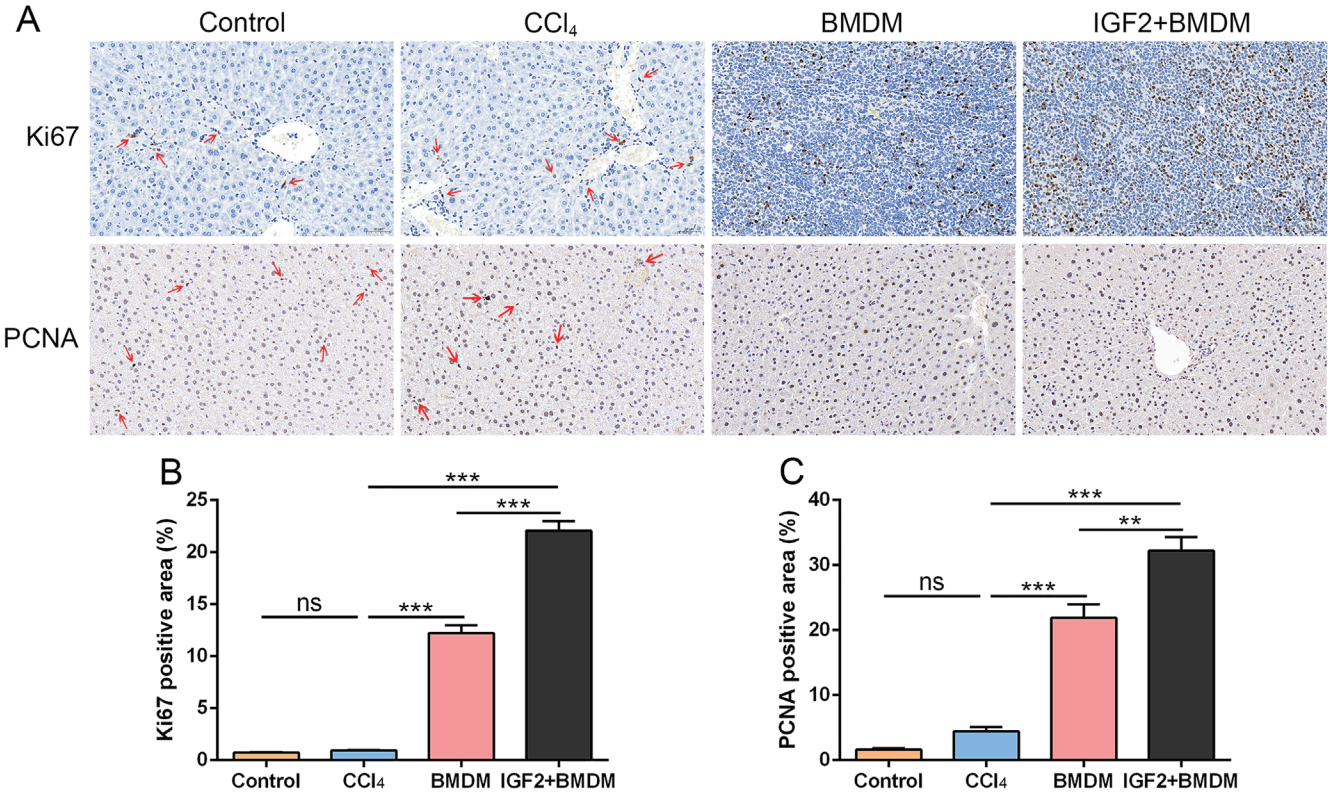

Fig. 3. Combining IGF2 with BMDM promotes hepatocyte regeneration in cirrhotic mice. (A) Immunostaining of Ki67 and PCNA in liver sections of mice in each group, arrows indicate Ki67- or PCNA-positive cells, scale: 50 μm. Quantification of Ki67-positive area (B) and PCNA-positive area (C). *p<0.05, **p<0.01, ***p<0.001; ns, not significant; BMDM, bone marrow-derived macrophages; IGF2, insulin-like growth factor 2; PCNA, proliferating cell nuclear antigen.

the nucleus (Fig. 4L). Therefore, we speculate that IGF2 may upregulate the expression of NR4A2 by activating the ERK1/2 pathway, thus promoting macrophage polarization toward an anti-inflammatory phenotype.

IGF2-NR4A2 pathway inhibits the activation of HSCs by mediating macrophage polarization toward the M2 subtype

First, we activated LX-2 HSCs by adding 5 ng/mL TGF-β1 for 24 h and induced the differentiation of THP-1 cells to M0, M1, and M2 macrophages, which were then co-cultured with the activated LX-2. The western blot results showed that when LX-2 was co-cultured with M0 macrophages, the protein expression levels of α-SMA and Collagen-1 were reduced, and the reduction was more pronounced after treatment with IGF2 (Fig. 5B–D). When LX-2 was co-cultured with M1 macrophages, the expression of Collagen-1 was increased, and α-SMA showed no significant difference. After treatment with IGF2, no significant difference was noted in the expression of α-SMA and Collagen-1 (Fig. 5E–G). When LX-2 was co-cultured with M2 macrophages, the protein expression levels of α-SMA and Collagen-1 were reduced, and the reduction was more obvious after treatment with IGF2 (Fig. 5H–J). To further verify the effects of IGF2 on the co-culture of macrophages and LX-2, we added the IGF2-neutralizing antibody Xentuzumab to the co-culture system, which partially counteracted the IGF2-induced inhibition (Fig. 5K–M). The results show that both M0 and M2 macrophages inhibited LX-2 activation, and that IGF2 enhanced the inhibition. To investigate the direct effect of IGF2 on LX-2, we added IGF2 to the medium of LX-2 and found no significant difference in the expression of α-SMA and Collagen-1 (Fig. 6A–C), indicating that IGF2 did not inhibit the activation of LX-2 directly but inhibited inducing M2 macrophage polarization.

IGF2-NR4A2 pathway alleviates liver cirrhosis by inducing matrix metalloproteinases in macrophages

The effective step to achieve fibrosis reversibility is degrading the excessive ECM. MMPs are a series of zinc-dependent ECM remodeling endopeptidases that play a critical role in ECM degradation and fibrosis regression.[25] We detected the expression of MMP2, MMP12, and MMP13 in liver tissue from mice in all groups, and the results showed that compared with the CCl_4 group, the IGF2 + BMDM group showed a significant increase in the protein expression levels of MMP12 and MMP13 (Fig. 1E, I, J). However, there was no significant difference in the expression of MMP2 among the groups (data not shown). Moreover, the in vitro experiments demonstrated that the protein expression levels of MMP2, MMP12, and MMP13 in macrophages were significantly increased after treatment with IGF2, and Xentuzumab could partially offset the increased MMPs (Fig. 6D–G). These results support the alleviation of liver cirrhosis by IGF2 promotion of MMP synthesis in macrophages, thus degrading ECM.

Overexpression of IGF2 significantly inhibits the activation of HSCs.

To confirm the role of IGF2 in macrophage polarization, we overexpressed IGF2 in THP-1 cells using lentiviral vectors. Transfection efficiency was detected by western blotting (Fig.

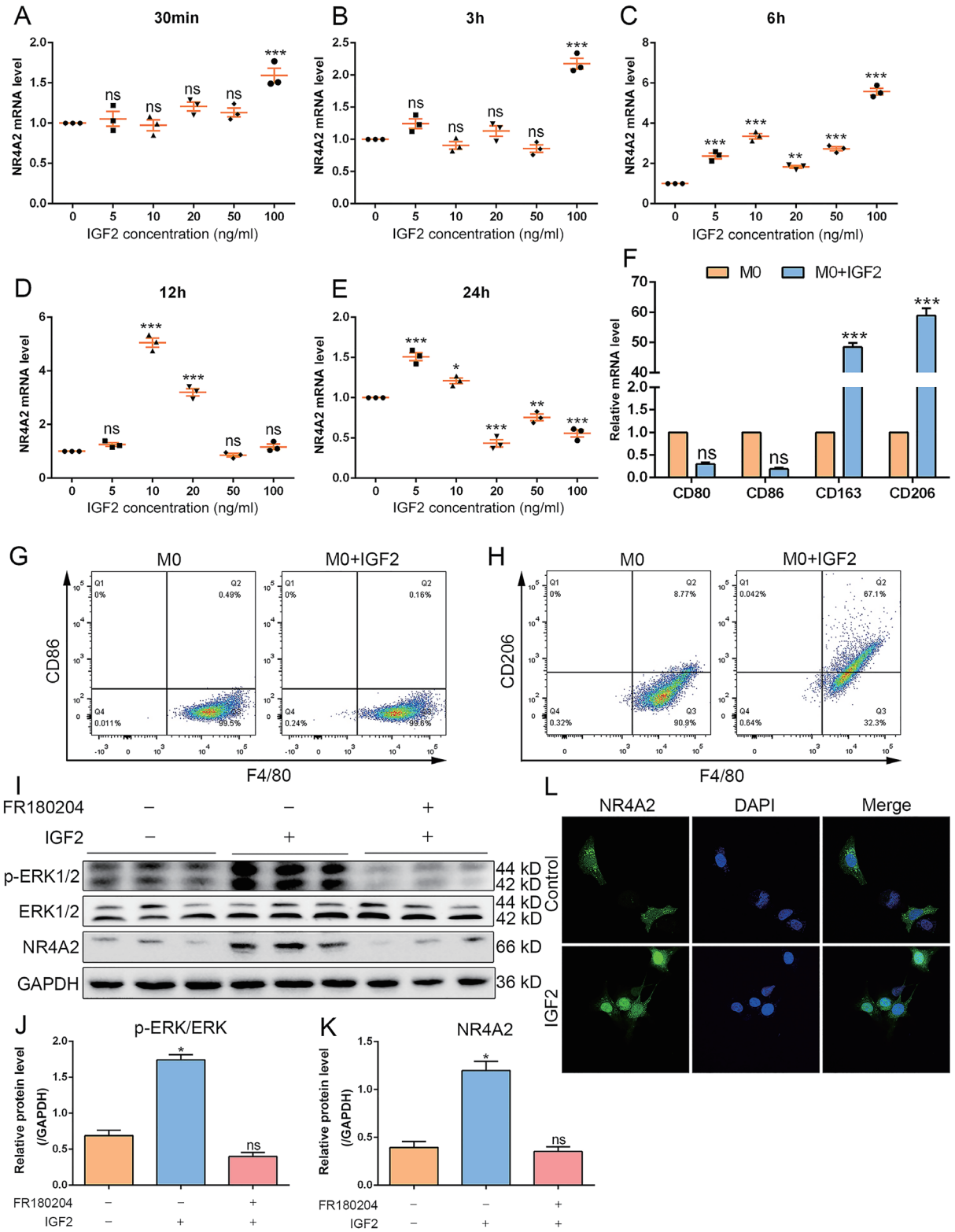

Fig. 4. The IGF2-NR4A2 pathway mediates macrophage polarization toward an anti-inflammatory phenotype. (A–E) mRNA expression of NR4A2 at 30 min, 3 h, 6 h, 12 h, and 24 h. (F) qRT-PCR showed low expression of the M1 markers CD80 and CD86, and high expression of the M2 markers CD163 and CD206. The percentage of $CD86^+$ cells (G) and $CD206^+$ cells (H) was determined by flow cytometry. (I–K) Western blotting was used to detect the activation of ERK1/2 pathway and the protein expression of NR4A2. (L) Cellular immunofluorescence shows translocation of NR4A2 expression to the nucleus. *p<0.05, **p<0.01, ***p<0.001; ns, not significant. IGF2, insulin-like growth factor 2; NR4A2, nuclear receptor family 4A, member 2.

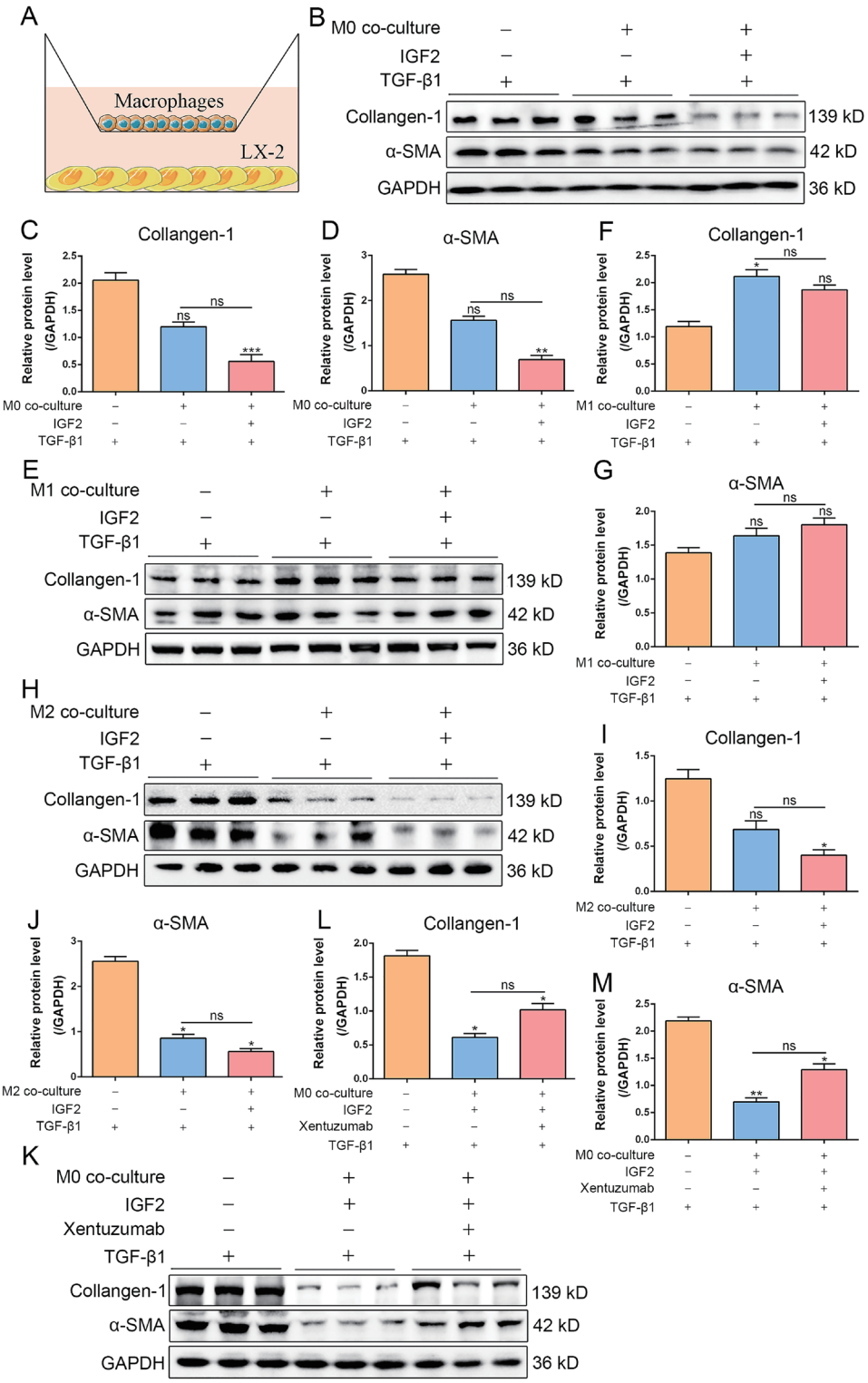

Fig. 5. The IGF2-NR4A2 pathway inhibits the activation of the HSCs by mediating macrophage polarization toward the M2 subtype. (A) Schematic shows the co-culture of macrophages and LX-2. (B–D) M0 macrophages were co-cultured with LX-2, and the protein expression of collagen-1 and α-SMA were determined by western blotting in the presence or absence of IGF2. (E–G) M1 macrophages were co-cultured with LX-2, and the protein expression of collagen-1 and α-SMA were determined by western blotting in the presence or absence of IGF2. (H–J) M2 macrophages were co-cultured with LX-2, and the protein expression of Collagen-1 and α-SMA were determined by western blotting in the presence or absence of IGF2. (K–M) IGF2 was co-cultured with M0 macrophages and LX-2, and the protein expression of collagen-1 and α-SMA were determined by western blotting in the presence or absence of Xentuzumab. *p<0.05, **p<0.01, ***p<0.001; ns, not significant. IGF2, insulin-like growth factor 2; NR4A2, nuclear receptor family 4A, member 2; HSCs, hepatic stellate cells; α-SMA, α-smooth muscle actin; TGF-β1, transforming growth factor beta 1.

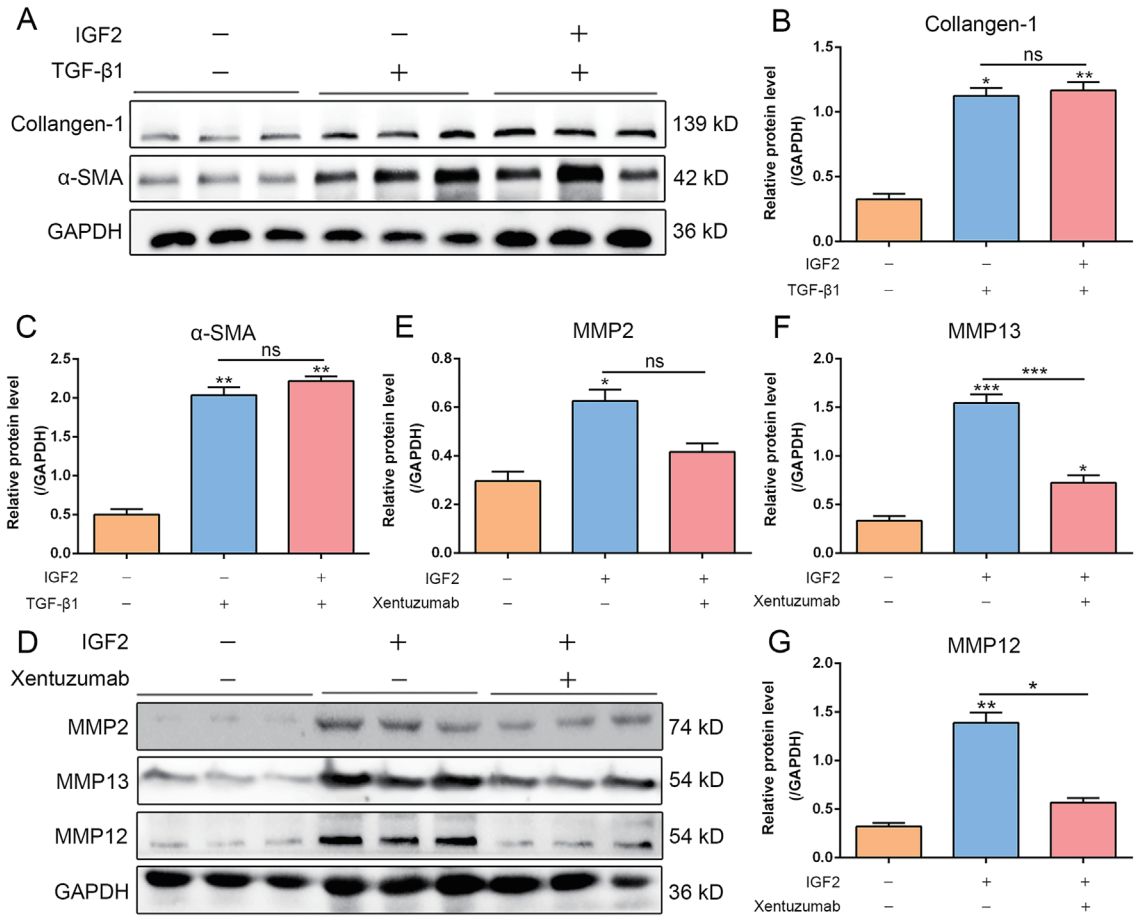

Fig. 6. The IGF2-NR4A2 pathway alleviates liver cirrhosis by inducing matrix metalloproteinases in macrophages. (A–C) LX-2 was treated with IGF2, and the protein expression of collagen-1 and α-SMA were determined by western blotting. (D–G) After treatment with IGF2, the protein expression of MMP2, MMP12, and MMP13 were determined by western blotting in the presence or absence of Xentuzumab. *p<0.05, **p<0.01, ***p<0.001; ns, not significant. IGF2, insulin-like growth factor 2; NR4A2, nuclear receptor family 4A, member 2; α-SMA, α-smooth muscle actin; TGF-β1, transforming growth factor beta 1; MMP2, matrix metalloproteinase 2; MMP12, matrix metalloproteinase 12; MMP13, matrix metalloproteinase 13.

7A, B). Compared with the control group, the protein expression level of IGF2 in the OE-IGF2 group was significantly increased, while no significant difference between the LV-NC and the control groups was noted. Additionally, we found that the expression level of NR4A2 in macrophages after overexpressing IGF2 was significantly increased in the OE-IGF2 group compared with the control group. Next, we induced the differentiation of transfected THP-1 cells into macrophages, which were then co-cultured with LX-2. The results showed that the protein expression levels of α-SMA and Collagen-1 in the LV-NC and OE-IGF2 groups were significantly reduced compared with the control group. The expression of α-SMA and collagen-1 in the OE-IGF2 group was further decreased, compared with the LV-NC group (Fig. 7C, D). Then, we examined the polarity of macrophages. The qRT-PCR assay showed that the expression levels of M2 macrophage markers CD163 and CD206 were significantly increased (Fig. 7E). The results indicate that IGF2 promoted macrophage polarization toward the M2 phenotype by upregulating NR4A2, thus inhibiting HSCs activation.

Discussion

We performed *in vivo* and *in vitro* experiments to discover novel information on how the IGF2-NR4A2 signaling pathway mediates macrophage polarization toward the M2 phenotype to alleviate liver inflammation and the development of liver cirrhosis. Macrophages are a heterogeneous population in the liver that promotes the development of inflammation and fibrosis and participate in the regeneration response after liver injury.[26] Macrophage-based cell therapy has been tested in different diseases, including liver diseases, but limited evidence exists supporting that macrophage therapy can alleviate liver fibrosis and activate liver regeneration.[7] Our results showed that combining IGF2 with BMDM reduced liver inflammation and fibrosis and increased hepatocyte proliferation in CCl_4-induced cirrhotic mice, and was more effective than treatment with BMDM alone. The *in vitro* experiments revealed that IGF2 promoted macrophage polarization toward the M2 phenotype by upregulating NR4A2 and exerting an anti-inflammatory effect to inhibit the activation of HSCs. Additionally, increased secretion of MMPs by M2 macrophages could degrade excessive ECM and play an anti-fibrosis role.

During the progression of liver fibrosis caused by various etiologies, proinflammatory cytokines released from liver-resident Kupffer cells in the initial phase, promote infiltration of immune cells. Inflammation further activates HSCs

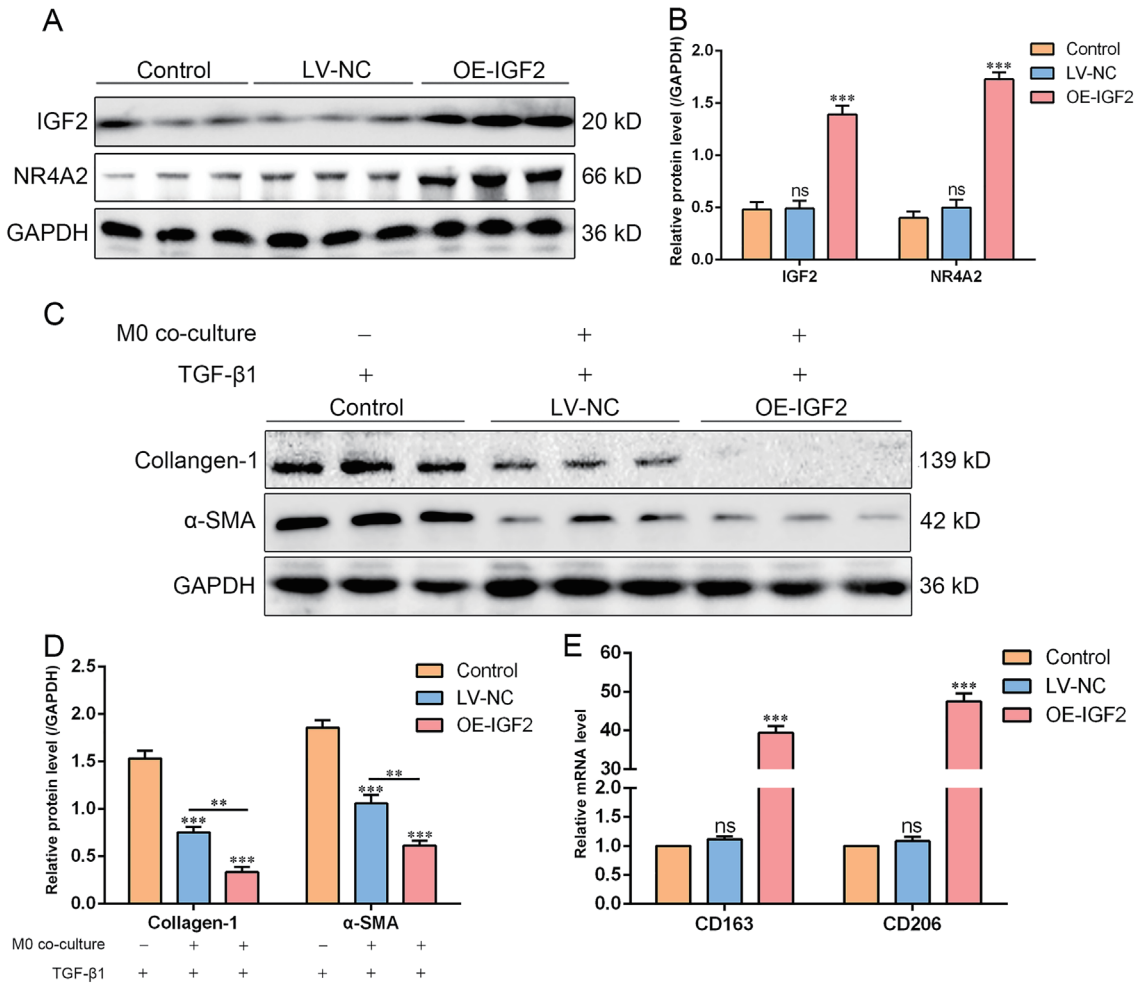

Fig. 7. Overexpression of IGF2 significantly inhibits the activation of hepatic stellate cells. (A–B) Protein expression of IGF2 and NR4A2 was determined by western blotting after transfection of THP-1 cells with lentivirus. (C–D) Macrophages were co-cultured with LX-2, and the protein expression of collagen-1 and α-SMA were determined by western blotting. (E) The mRNA levels of M2 markers CD163 and CD206 were assayed by qRT-PCR after overexpression of IGF2. *p<0.05, **p<0.01, ***p<0.001; ns, not significant. IGF2, insulin-like growth factor 2; NR4A2, nuclear receptor family 4A, member 2; α-SMA, α-smooth muscle actin; TGF-β1, transforming growth factor beta 1.

to transform from quiescent retinyl ester-storing cells to collagen and other ECM-producing myofibroblasts that are involved in liver fibrosis and the reconstruction of intrahepatic structures.[27] Activated HSCs also produce many chemokines and cytokines, which promote the development of liver inflammation and fibrosis.[28,29]

IGF is an autocrine/paracrine growth factor essential for many physiological functions, including cell growth, proliferation, metabolism, and glucose homeostasis.[30] Recent studies have shown that IGF2 attenuated EAE and DSS-induced inflammatory bowel disease in mice by reprogramming macrophages to acquire an M2 anti-inflammatory phenotype.[18,19] Previous studies have demonstrated the therapeutic effects of M2 macrophages in mice with liver fibrosis. For example, proline-serine-threonine-phosphatase-interacting protein 2 (PSTPIP2) stimulated M2 macrophage polarization in a STAT1-STAT6-dependent manner, which eased liver inflammation and fibrosis in mouse models of CCl_4-induced liver fibrosis.[31] Maresin-1 regulated macrophage polarization toward the M2 subtype via retinoic acid-related orphan receptor α (RORα)/12-lipoxygenase (12-LOX) to counteract inflammation and fibrosis in mice receiving a high-fat diet (HFD).[32] Those studies prompted us to speculate whether IGF2 had anti-inflammatory and anti-fibrotic activity in the liver that induced macrophage M2 polarization.

Our study showed that administering BMDM only and IGF2 combined with BMDM to mice with CCl_4-induced liver cirrhosis improved the degree of cirrhosis, and the protein expression levels of the fibrosis marker genes α-SMA, Collagen-1, and TGF-β1 were significantly reduced. Moreover, the reduction in their expression was more pronounced in the IGF2 + BMDM group than in the BMDM group. In the co-culture of macrophages and LX-2, we found that both M0 and M2 macrophages suppressed the expression levels of α-SMA and Collagen-1, with M2 macrophages showing a stronger suppressive effect. This is consistent with previous findings. When LX-2 was co-cultured with M0 macrophages, the expression of α-SMA and Collagen-1 significantly decreased in the presence of IGF2. The IGF2-neutralizing antibody Xentuzumab, partially reversed the response to LX-2, which demonstrated the anti-fibrosis activity of IGF2-treated macrophages. Next, we determined the polarity of macrophages after IGF2 intervention. qRT-PCR indicated high expression of the M2 markers, CD163 and CD206, and low expression of the M1 mark-

ers, CD80 and CD86. Flow cytometry revealed a significantly increased percentage of $CD206^+$ cells. The results show that IGF2 promoted macrophage polarization toward M2, and the anti-inflammatory activity inhibited LX-2 activation, thereby alleviating liver cirrhosis.

NR4A is aberrantly expressed under inflammatory conditions and participates in cellular processes involving chronic inflammation. It is a potential regulator of various cytokines and growth factors, modulating the inflammatory response in several diseases.[33] For example, NR4A2 has a crucial role in the growth and maintenance of dopaminergic neurons and antineuronal inflammation in Parkinson's disease.[34] Xu *et al*.[35] reported that the reduced expression of NR4A2 was associated with chronic inflammation in patients with type 2 diabetes. Furthermore, NR4A2 promoted M2 polarization to reduce the inflammatory response of sepsis.[36] Miao *et al*.[37] reported that overexpression of NR4A2 increased M2 macrophages and attenuated myocardial injury and fibrosis in rat heart tissue. Considering the importance of NR4A2 in inflammatory diseases and macrophage polarization, we tested whether IGF2 induced macrophage M2 polarization by regulating NR4A2. We found that IGF2 significantly increased the expression of NR4A2 in macrophages by activating the ERK1/2 pathway, thus inducing macrophages to polarize toward the M2 subtype, resulting in anti-inflammatory activity that inhibited the activation of LX-2. To further verify the regulatory effect of IGF2 on macrophages, we overexpressed IGF2 in macrophages using a lentivirus vector and discovered that the expression level of NR4A2 was also significantly increased. When the above cells were co-cultured with LX-2, the expression levels of α-SMA and Collagen-1 in LX-2 were significantly reduced. qRT-PCR showed that expression of the of the M2 macrophage markers CD163 and CD206 was significantly increased after the overexpression of IGF2, showing that IGF2 induced macrophage M2 polarization by upregulating NR4A2.

MMPs have an essential role in ECM remodeling and fibrosis regression. Studies have shown that when chronic injury stops, local molecular signaling triggers the transformation of M1 macrophages into M2 macrophages, which contributes to the regression of fibrosis by producing specific MMPs and other protein hydrolases (e.g., histone proteases) to degrade the ECM.[38–40] Therefore, we examined the expression level of MMPs in liver tissue from mice and found that MMP12 and MMP13 were significantly increased in both the BMDM and IGF2 + BMDM groups, with higher expression in the latter. Similarly, IGF2 enhanced MMP2, MMP12, and MMP13 expression in macrophages *in vitro*, which was partially offset by Xentuzumab. This proved that IGF2 could promote the production of MMPs in macrophages to degrade ECM, thereby alleviating liver cirrhosis.

It has been reported that production of IGF2 by pericentral hepatocytes promoted hepatocyte proliferation and repair of damaged tissue during chronic liver injury.[41] In this study, the immunohistochemical results showed that the proliferation markers Ki67 and PCNA were significantly increased in both the BMDM and IGF2 + BMDM groups, with the latter having a higher expression level. Unfortunately, our study did not have an IGF2-only group and ignored the effect of the administration IGF2 alone on mouse hepatocyte proliferation. Additionally, we used only one mouse cirrhosis model in this study and could not illustrate the role of the IGF2-NR4A2 pathway in different liver injury models. It is necessary to construct more mouse liver cirrhosis models (e.g., bile duct ligation) in the future to further explore the role of the IGF2-NR4A2 pathway in different internal environments of liver injury.

Conclusion

In conclusion, IGF2 promoted macrophage polarization toward an anti-inflammatory phenotype by upregulating NR4A2, thus inhibiting HSCs activation and attenuating liver inflammation and fibrosis. That provides a theoretical basis for the future use of BMDM-based cell therapies for liver cirrhosis. However, given the complexity of immune and inflammatory responses under pathological conditions and the fact that cirrhosis in humans differs from that in mice models, further studies on the interaction between macrophages and host cells are needed to better understand the safety and efficacy of macrophage therapy for liver cirrhosis before any potential clinical application.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Conceived and designed the study (YJ, KD), designed the research studies, conducted experiments, acquired data, and analyzed data (LY, XH), drafted the manuscript (LY, KD), conducted experiments, acquired data, and provided reagents (MY, QZ, PL, LY). All authors contributed to the article and approved the submitted version.

Ethical statement

The animal study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Animal Care and Use Committee of Renmin Hospital of Wuhan University (No. 20211204).

Data sharing statement

The datasets generated for this study are available from the corresponding author on reasonable request.

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