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Effect of microRNA-155 on the Interferon-Gamma Signaling Pathway in Biliary Atresia

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Abstract

MicroRNAs (miRNAs) are ~22-nucleotide long RNAs that negatively regulate gene expression and inflammatory responses in eukaryotes. The aim of this work was to evaluate the roles of miRNA (miR)-155 on the interferon-γ (IFN-γ)-induced response in biliary atresia (BA), which is the most common form of pediatric chronic liver disease and a leading indication for pediatric liver transplantation. The expression of miR-155 and the suppressor of cytokine signaling 1 (SOCS1) gene in human and mice liver tissues of BA and healthy controls was evaluated. IFN-γ-induced expression of miR-155, inflammatory cytokines and chemokines was determined in bile duct cells. A miR-155 inhibitor was used to determine the influence in the IFN-γ-induced signaling pathway by western blot analysis. A strong up-regulation of miR-155 expression was observed in BA histologic sections and mouse bile duct cells treated with IFN-γ. miR-155 down-regulated SOCS1 protein expression by targeting its mRNA. Up-regulation of miR-155 expression by IFN-γ in bile duct cells led to the activation of signal transducers and activators of transcription 1 (Stat1) and inflammatory cytokines through the Janus kinase (Jak)/Stat pathway, whereas targeted inhibition of miR-155 expression by anti-miRNA oligonucleotides significantly decreased the mRNA or protein expression levels of these inflammatory cytokines and Stat1. Overall, our results suggest that miR-155 regulates the IFN-γ signaling pathway by targeting SOCS1 expression and may be a potential target in BA therapy

Key Words: bile duct, inflammatory response, interferon gamma, Jak/Stat pathway, microRNA-155, SOCS1

Introduction

Biliary atresia (BA) is the most common form of chronic liver disease in children and the leading indication for pediatric liver transplantation worldwide (2). BA occurs when a primary injury of unknown etiology leads to progressive T cell-mediated destruction of the extrahepatic biliary system (15). The pathology of early BA includes the presence of extrahepatic bile ducts (EHBD) with inflammation and fibrosis in the hepatic portal area (33). Approximately 70-80% of BA patients eventually require liver transplanta-

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tion, attesting to the need for a better understanding of the underlying etiology and pathogenesis of this disease (27). Previous studies identified the signatures of overexpression of interferon-gamma (IFN-γ) and other T lymphocyte-enriched genes in the liver (3). In addition to the tissue-specific population of T lymphocytes in virus-infected mice, livers exhibited significantly increased levels of IFN-γ and IL-12p40 (3). IFN-γ has been implicated as an important effector in the pathogenesis of autoimmune disorders. While the virus-inducible inflammatory response progresses to complete ductal obstruction in wild-type (WT) BALB/c mice, loss of IFN-γ prevents excessive temporospatial accumulation of lymphocytes and obstruction of extrahepatic ducts (34). These findings demonstrate that IFN-γ may play a key regulatory role in the pathogenesis of bile duct injury and obstruction in BA. The presence of excess extracellular IFN-γ suggests the existence of additional mechanisms to modulate the extent of ligand stimulation by IFN-γ signaling (8, 12, 31). Suppressor of cytokine signaling 1 (SOCS1) is a specific feedback inhibitor of IFN-γ that is associated with Jak1/2, thereby interfering with tyrosine kinase activity and inhibiting downstream IFN-γ signaling (14, 19). The role IFN-γ on the pathogenesis of BA still needs to be investigated. MicroRNAs (miRNAs) are single-stranded small (~22 nucleotides) noncoding RNA molecules, which post-transcriptionally regulate gene expression by targeting mRNAs for degradation and translational repression; miRNAs are also known to regulate inflammatory responses (20). A specific miRNA, miR-155, is involved in cell proliferation, inflammation, differentiation and apoptosis (21, 28). The gene encoding miR-155 and its precursor noncoding transcript *BIC* is localized to the human chromosome band 21q21.3 (11). Several genes, including activation-induced cytidine deaminase (AICDA), Src homology 2 domain-containing inositol-5-phosphatase 1 (SHIP1), CCAAT/enhancer-binding protein beta (CEBPB), SPI1 proto-oncogene and SOCS1, are targeted for translational modulation by miRNA miR-155 (6, 9, 35). By targeting SOCS1, miR-155 regulates antiviral innate immunity, dendritic cell development, modulates macrophage response to lipopolysaccharide, controls osteoclast differentiation and acts as an oncogene in breast cancer (10). Recent studies have indicated that inflammatory mediators such as IFN-γ can induce miR-155 expression in retinal pigment epithelial cells, or in breast cancer cells (17, 20). However, to the best of our knowledge, the potential role of miR-155 in modulating inflammatory responses in BA cases by targeting SOCS1 has not been determined. Therefore, we investigated expression of miR-155 in BA in response to treatment with the inflammatory cytokine IFN-γ.

Materials and Methods

Tissues and Cell Culture

Human BA and normal specimens were collected from patients in Kaohsiung Medical University Chung-Ho Memorial Hospital (KMUH) in accordance with the approved guidelines by the Hospital Ethics Committee of KMUH. Informed consent was taken from each participant. We collected 20 liver tissues of BA patients and 5 liver tissues without BA and liver diseases as controls. Neonatal C57BL/6 mice were injected intraperitoneally with 10^6 plaque-forming units of rotavirus (5 mice), or with saline (5 mice), in the first 24 h of birth. Mice were killed at 7 or 14 days after injection of the virus or a saline solution (11). At the time of death, the EHBDs and gallbladder were microdissected and fixed in formaldehyde. All tissues were paraffin-embedded and sectioned, and total RNA extracted for quantitative polymerase chain reaction (qPCR) analysis. Primary bile duct cells were cultured from 8 week-old mice. In brief, bile ducts were removed, added to Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), and triturated using trypsin. Dissociated cells were then plated in 10-cm dishes and cultured for 1-2 weeks. After culturing, 2×10^5 cells were seeded into 6-cm dish and incubated with 50 ng/ml recombinant murine IFN-γ for 0.5, 2, 6, or 12 h. The animal studies were maintained in a pathogen-free barrier facility and the experiments were carried out in accordance with China Medical University (CMU) approved protocol with relevant guidelines maintained by the committee.

Transfection of miRNA Inhibitor

Bile duct cells were maintained at~80% confluence in 6-cm dishes. Cells were transiently transfected with 5′FAM-modified miR-155 inhibitor (GeneDireX, Irvine, CA, USA; 5'- ACCCCUAUCACGAUUAGCAUUAA -3'), or a negative control (NC) inhibitor (5'-CAGUACUUUUGUGUAGUACAA -3'), using Lipofectamine 2000 (Invitrogen, Waltham, MA, USA) for 6-8 h according to the manufacturer's instructions.

Real-Time qPCR Assay

Total RNA and miRNA were isolated using a High Pure miRNA Isolation Kit (Roche, Penzberg, Bavaria, Germany), and cDNA was prepared using a High-Capacity cDNA Reverse Transcriptase Kit (ABI, Waltham, MA, USA) according to the manufacturers' instructions. Levels of SOCS1, chemokine (C-X-C motif) ligand 9 (CXCL9), CXCL10, interleukin-18 (IL-18), IL-1α and IL-1β transcripts were

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Table 1. Sequences of qPCR primers

Gene	Primer	Sequence 5' – 3'
SOCS1	Forward	5′-GTGGTTGTGGAGGGTGAGAT-3′
SOCS1	Reverse	5′-CCTGAGAGGTGGGATGAGG-3′
CXCL9	Forward	5′-CTTTTCCTCTTGGGCATCAT-3′
CXCL9	Reverse	5′-GCATCGTGCATTCCTTATCA-3′
CXCL10	Forward	5′-GCTGCCGTCATTTTCTGC-3′
CXCL10	Reverse	5′-TCTCACTGGCCCGTCATC-3′
IL-18	Forward	5′-CAAACCTTCCAAATCACTTCCT-3′
IL-18	Reverse	5′-TCCTTGAAGTTGACGCAAGA-3′
IL-1a	Forward	5′-TTGGTTAAATGACCTGCAACA-3′
IL-1a	Reverse	5′-GAGCGCTCACGAACAGTTG-3′
IL-1b	Forward	5′-TGTAATGAAAGACGGCACACC-3′
IL-1b	Reverse	5′-TCTTCTTTGGGTATTGCTTGG-3′
GAPDH	Forward	5′-GCCAAAAGGGTCATCATCTC-3′
GAPDH	Reverse	5′-CACACCCATCACAAACATGG-3′

measured by real-time qPCR on an LC480 (Roche) using a universal probe system (UPL Roche, Penzberg, Bavaria, Germany). Forward and reverse primers were designed by Universal ProbeLiberary Design Center (Roche). The sequences of primers were displayed in Table 1. Levels of miR-155 and the housekeeping miRNA RNU6B were measured by qPCR. miR-155 and RNU6B primers were purchased from ABI. The threshold cycle number was calculated for each gene and normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or RNU6B. The ΔC_t values for each gene are presented as relative fold induction.

Western Blot Analysis

The cells were washed twice with cold phosphate buffered saline (PBS) and lysed with cell lysis radioimmunoprecipitation assay (RIPA) buffer supplemented with a protease inhibitor mixture (Roche). The protein concentrations of the cell lysates were measured using a bicinchoninic acid (BCA) assay (BioRad, Hercules, CA, USA) and equalized with the extraction reagent. Equal amounts of the extracts were loaded and subjected to SDS-PAGE, transferred onto 0.2 μm poly-vinylidene difluoride (PVDF) membranes (Millipore, USA), and stained with appropriate antibodies (anti-SOCS1: Abcam, UK; tyrosine kinase 2 (Tyk2), phosphor-Tyk2, signal transducers and activators of transcription 1 (Stat1), phospho-Stat1 and beta-actin: Cell Signaling, Beverly, MA, USA). The reaction was visualized by chemiluminescence.

Enzyme-Linked Immunosorbent Assay (ELISA) Analysis

Approximately 2×10^5 cells in 5 mL culture medium were seeded into 6-cm dishes, incubated overnight, and transfected as described above. After 24 h, the cells were treated with IFN-γ for the indicated times. The concentrations of IL-6, monocyte chemoattractant protein-1 (MCP-1) and CXCL1 in culture supernatants were measured using ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. All of the samples were stored at -20°C before further assays.

Statistical Analysis

All data presented in this study were analyzed using Student's *t*-test.

Results

Expression of miR-155 Was Significantly Upregulated in BA Tissues and Cells

To explore the role of miR-155 in the pathogenesis of BA, liver tissue samples were collected from patients with BA and healthy controls in both humans and mice. qPCR was then used to measure the miR-155 expression levels. Expression of miR-155 in BA tissues was significantly higher than that in the controls (Fig. 1A). To further confirm the roles of miR-155 in BA, the expression of miR-155 in mouse bile duct cells under IFN-γ treatment was analyzed, and a 19-fold higher miR-155 expression was found after IFN-γ treatment for 2 h (Fig. 1B). IFN-γ activated Janus kinase (Jak)/Stat signaling pathway in bile duct cells, which may participate in the up-regulation of miR-155. Time-dependent IFN-γ-induced Stat1 expression was observed (Fig. 1C). To elucidate the response of inflammatory cytokines in bile duct cells under IFN-γ treatment, secretion of several cytokines and chemokines were analyzed. Increased secretion of CXCL1, IL-6 and MCP-1 from bile duct cells was observed (Fig. 1D). These results suggest that IFN-γ treatment induces miR-155 expression in bile duct cells and promotes an inflammatory response.

SOCS1 Is a Target of miR-155 in BA Tissues

To identify the potential targets of miR-155 that might be involved in the BA inflammatory response, we next screened the mouse and human miR-155 sequences using the miRBase target identification program. Among other important targets such as Ras homolog gene family member A (RhoA), Fos, Myb and the receptor-interacting protein kinase (21), SOCS1 was identified as a potential target of miR-155

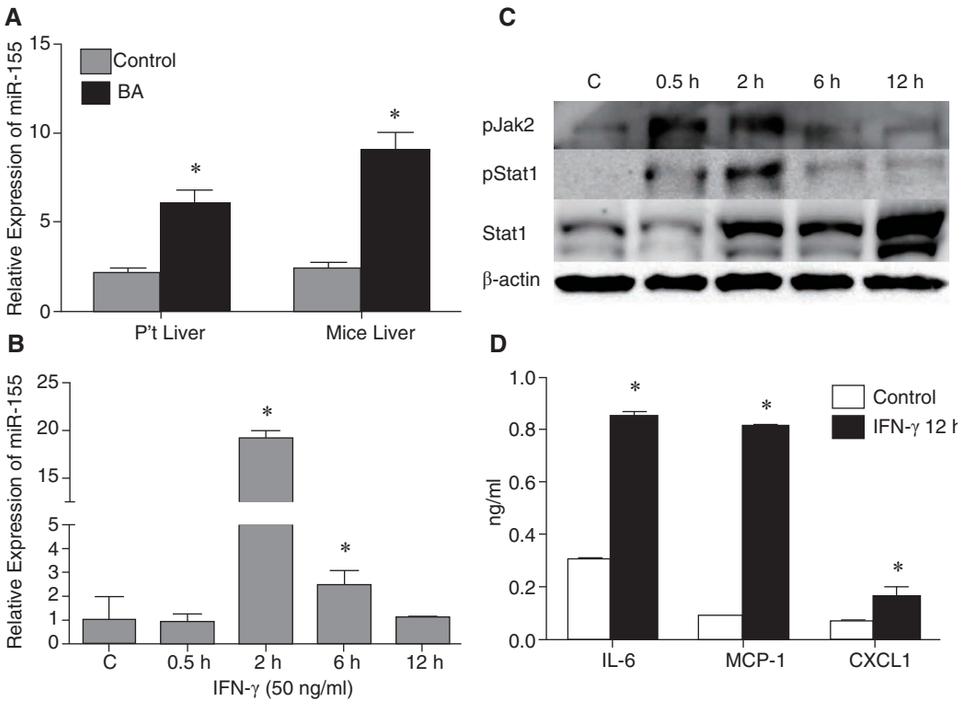

Fig. 1. Expression of miR-155 in and cytokine secretion by BA tissues and cells. (A) Analysis of miR-155 expression by qPCR in human and mouse liver tissues with BA compared to that in normal liver samples. (B) Bile duct cells were treated with IFN-γ (50 ng/mL) for 0.5, 2, 6, or 12 h, followed by qPCR analysis. Fold changes in expression levels are shown. (C) WB analysis of IFN-γ induction of the Jak/Stat signaling pathway. (D) Response of bile duct cells to treatment with IFN-γ for 12 h by markedly increasing the secretion of CXCL1, IL-6 and MCP-1. The concentrations of cytokines and chemokines in the culture supernatants were estimated by ELISA. Results indicate the mean ± standard deviation of three independent experiments performed in triplicates. *$P < 0.05$ *vs*. control. P't Liver: patients' liver.

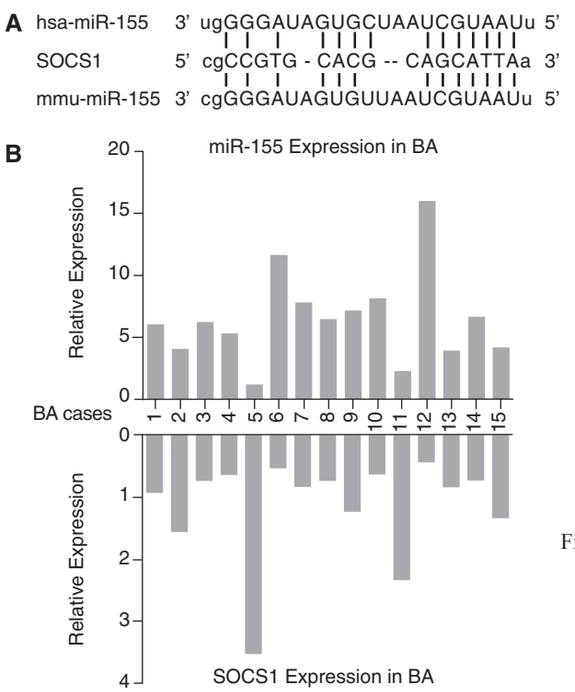

Fig. 2. miR-155 targets SOCS1. (A) SOCS1 was predicted to be a target of miR-155 in both human and mice by miRBase analysis. (B) miR-155 and SOCS1 mRNA levels in liver tissues were determined by qPCR in fifteen cases with BA.

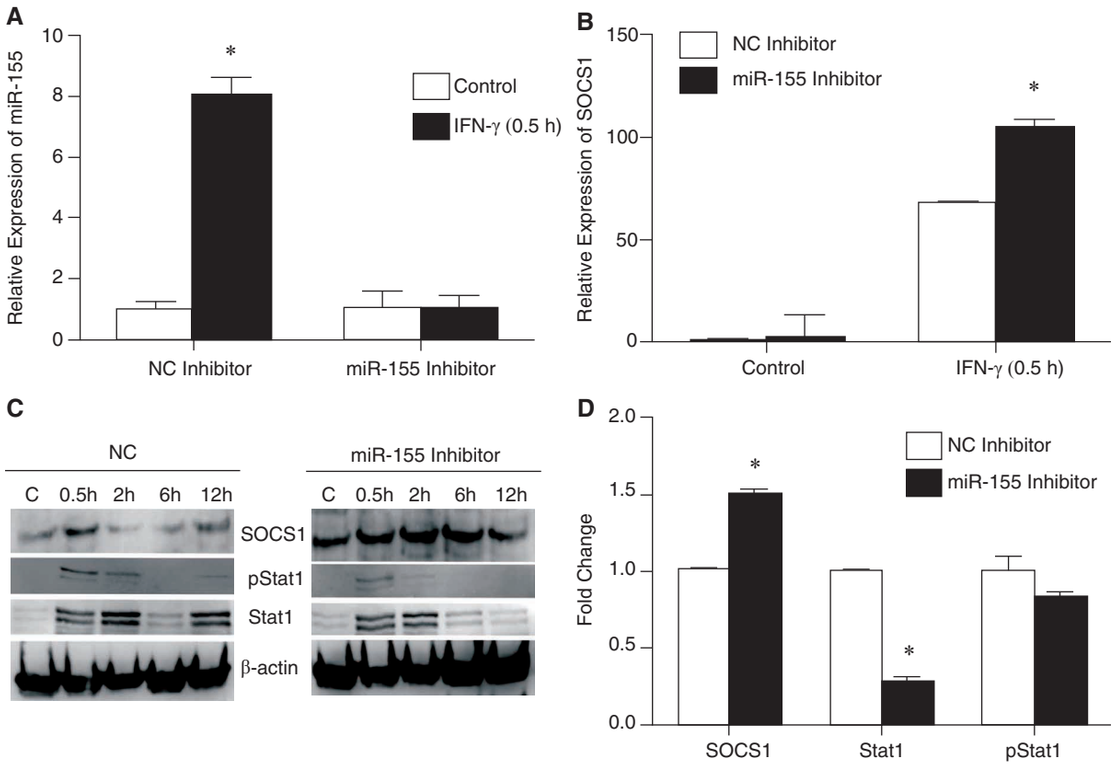

Fig. 3. miR-155 modulates the Jak/Stat signaling pathway. Bile duct cells were transfected with a NC inhibitor or miR-155 inhibitor. Twenty-four hours after transfection, cells were incubated with 50 ng/mL IFN-γ. (A) Expression levels of miR-155 was effectively blocked by the transfected miR-155 inhibitor. (B) Elevated SOCS1 expression levels in cells transfected with miR-155 inhibitor by qPCR. (C) Suppression of miR-155-increased SOCS1 and reduced Stat1 and Stat1 activation. Western blot analysis was performed at the indicated time after transfection. (D) The expression levels of SOCS1 and activated Stat1 were determined by densitometry analysis. Results indicate the mean ± standard deviation of three independent experiments performed in triplicates. *$P < 0.05$ *vs*. control (transfected with NC inhibitor).

in both humans and mice (Fig. 2A). To determine whether this observation could be extended to primary BA tissues, SOCS1 and miR-155 expression in 15 BA tissue specimens were examined. A significant ($P < 0.05$) inverse correlation between SOCS1 and miR-155 expression was found (Fig. 2B).

Effects of miR-155 Silencing on the Expression of Inflammatory Cytokines

Based on these observations, we measured miR-155 levels in mouse bile duct cells transfected with NC, or a miR-155 inhibitor, for 6-8 h and then treated with IFN-γ. The expression level of miR-155 was significantly down-regulated by the miR-155 inhibitor compared to the NC inhibitor ($P < 0.05$) (Fig. 3A). The miR-155 inhibitor was further found to increase IFN-γ-induced SOCS1 expression (Fig. 3, B to D). IFN-γ up-regulated expression of inflammatory cytokine genes CXCL10, IL-18, IL-1a, IL-1b and CXCL9 as determined by qPCR. On the other hand, expression of these cytokines was down-regulated upon miR-155 inhibition (Fig. 4A). Similarly, secretion of the inflammatory cytokines IL-6, MCP-1 and CXCL1 was also decreased upon miR-155 suppression (Fig. 4B). SOCS1 was previously reported to be associated with Jak and blocks its tyrosine kinase activity and downstream Stat phosphorylation (31). Our results further indicated that miR-155 regulated IFN-γ signaling *via* SOCS1. Inhibition of the inducible miR-155 suppressed Stat1 phosphorylation and the expression of the unphosphorylated form (Fig. 3, C and D). Taken together, these results suggest that bile duct cells exert an anti-inflammatory response by maintaining SOCS1 expression through regulation of miR-155 expression.

Discussion

MiR-155 is commonly represented as a multifunctional miRNA. It is transcribed from the noncoding

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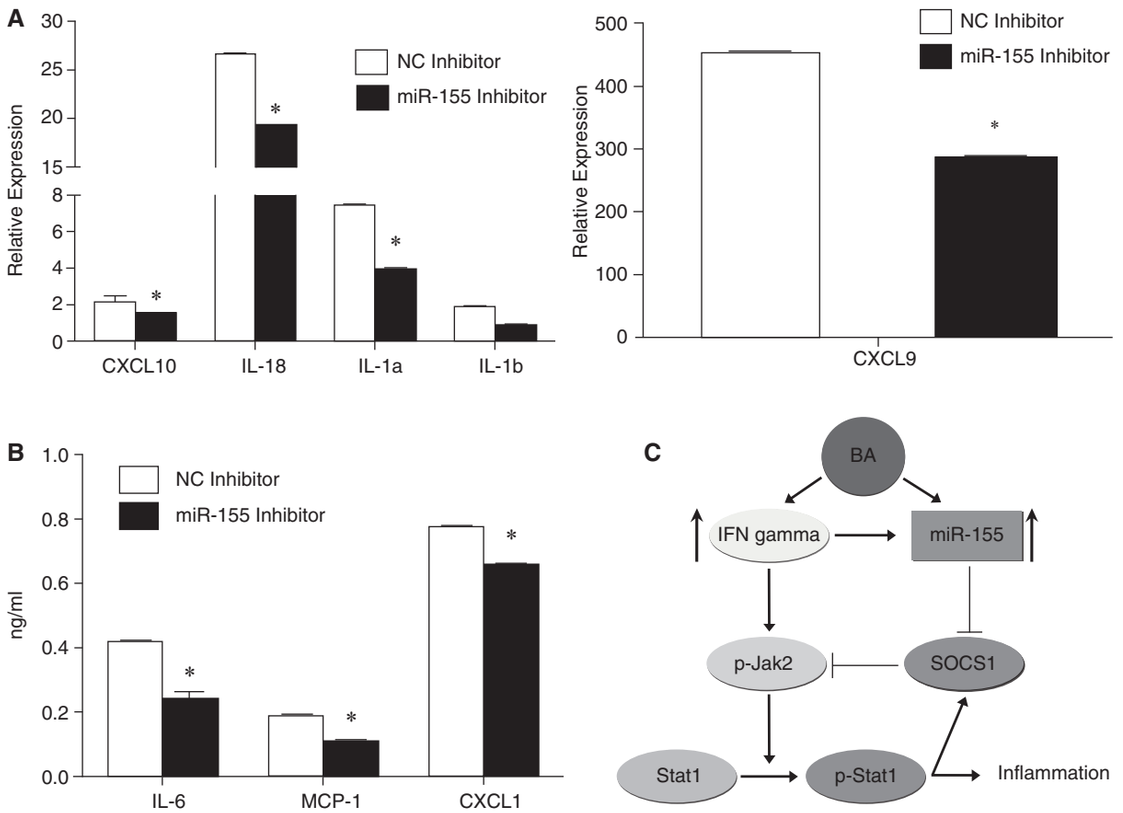

Fig. 4. Modulation of inflammatory cytokine expression by inhibition of miR-155. (A) The expression levels of CXCL10, IL-18, IL-1a, IL-1b and CXCL9 were determined by qPCR. Results are presented as the fold change after treatment with IFN-γ for 2 h. (B) The expression levels of IL-6, MCP-1 and CXCL1 were determined by ELISA. Results indicate the mean ± standard deviation of three independent experiments performed in triplicates. *$P < 0.05$ *vs.* control (transfected with NC inhibitor). (C) A proposed model of miR-155 in the pathogenesis of BA.

BIC gene, which is highly conserved in many species and plays a role in numerous biological processes including hematopoiesis, immunity and inflammation under physiological conditions (11). miR-155 expression has been demonstrated in several cell types, including macrophages, microglial cells, dendritic cells and hematopoietic progenitors (25). To our knowledge, this is the first study on the roles of miR-155 in the pathogenic mechanism of BA. Our results demonstrate that miR-155 is significantly up-regulated in both primary human and mouse tissues in BA (Fig. 1A). Furthermore, we found that miR-155 is also significantly up-regulated in bile duct cells following cell activation upon exposure to IFN-γ (Fig. 1B). These results indicate that IFN-γ induces miR-155 expression and that inhibition of miR-155 expression mitigates the IFN-γ-mediated activation of downstream inflammatory cytokines in bile duct cells, suggesting that miR-155 is a positive feedback regulator in anti-inflammatory responses.

IFN-γ is produced by cluster of differentiation 4+ (CD4+) T-helper cell type 1 (Th1) lymphocytes, CD8+ cytotoxic lymphocytes, NK cells, B cells and professional antigen-presenting cells (APCs), and is important in early host defense against infection in the adaptive immune response (12, 32). Infectious agents trigger IL-12 and IL-18 production by macrophages, which in turn act upon NK and T cells to promote IFN-γ synthesis. IFN-γ synthesis is also promoted by IL-1, IL-2, growth factors, estrogen and IFN-γ itself, and is inhibited by transforming growth factor-β (TGF-β), IL-4, IL-10 and glucocorticoids (13, 16, 30). IFN-γ primarily signals through the Jak/Stat pathway, which involves sequential receptor recruitment and activation of Jak and Stat to control the transcription of target genes. Signaling induces Jak2 autophosphorylation and activation, which allows Jak1 transphosphorylation by Jak2. Activated Jak1 recruits and phosphorylates Stat1 (4), which promotes dissociation of Stat1 from the receptor and its transport to the nucleus to initiate or suppress transcription of IFN-γ-regulated genes by binding to promoter elements (7). Previous studies

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showed that Stat1 activation is inhibited within 1 h of IFN-γ treatment (29). One of the targets of IFN-γ is a feedback inhibitor, SOCS1. SOCS1 can interfere with tyrosine kinase activity and inhibit IFN-γ signaling; SOCS1-knockout mice are hyper-responsive to infection and show enhanced macrophage activity (1, 23).

BA is a liver disease characterized by inflammatory and fibrotic obliteration of the EHBD, leading to cholestasis and biliary cirrhosis. The only therapies are Kasai portoenterostomy (KPE) and liver transplantation (26, 38). Loss of IFN-γ expression completely prevented inflammation and fibro-obstruction of bile ducts and that IFN-γ plays a pivotal regulatory role in the obstruction of EHBDs. IFN-γ has been shown to increase CXCL1 secretion and to modulate expression of CXCL9 and CXCL10 chemokines, and affects other cellular inflammatory cytokines, such as the IL-1 family, to drive the molecular network that is known to polarize lymphocytes to proinflammatory profile in bile duct cells (5, 18, 24). Previous studies have reported that increased Th17 cells as a new unique subpopulation of $CD4^+$ T cells may play a role in the pathogenesis of BA. Th17 cells exert their function by secreting IL-17α, which has a variety of effects, including induction of proinflammatory cytokines such as IL-23, IL-6, IL-8 and IL-1 as well as chemokines such as CXCL1, CXCL2, CXCL3, CXCL6 (GCP-2), CXCL8 (IL-8), CCL2 (MCP-1) and CCL (CC-chemokine ligand)-20, which mediate infiltration of tissue inflammatory cells (22, 36, 37). This suggests that promoting the production of these cytokines and chemokines is an important inflammatory processes in biliary injury of BA.

miRNAs can inhibit mRNA translation by specifically binding to the seed site in the 3'-untranslated region (3′UTR) of target transcripts (21). In our study, miR-155 expression was significantly higher in the BA group than in the control group. Increased miR-155 expression levels were observed in the BA model, which was similar to the expression levels previously observed in mouse macrophages (25). We also identified a predicted binding site of miR-155 in the SOCS1 3′UTR using miRBase (Fig. 2A). Suppression of miR-155 expression up-regulated SOCS1 expression in bile duct cells (Fig. 3B), inhibited the expression of inflammatory cytokines (Figs. 4A and B), and reduced Jak/Stat pathway signaling (Figs. 3, B to D). Moreover, we observed decreased protein levels of Stat1 after miR-155 suppression. A possible reason for this might be that activated Stat1 can bind to the Stat1 binding element in the *BIC*/miR-155 promoter (20). Furthermore, SOCS1 is a well-known negative feedback inhibitor of Stat1 activation (39). Hence, miR-155-suppressed SOCS1 expression would result in the enhancement of Stat1-induced inflammation.

Our results showed that IFN-γ markedly increased the expression of miR-155 in bile duct cells. We have also provided evidence that the Jak/Stat signaling pathway could be involved in the regulation of miR-155 expression (Fig. 4C). miR-155 has the potential to modulate the response of bile duct cells to inflammatory stimuli and, therefore, may serve as a novel target for therapeutic intervention in liver diseases.

Acknowledgments

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Conflict of Interests

The authors declare no competing financial interests.

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