miR-155 regulates pro- and anti-inflammatory cytokine expression in human monocytes during chronic hepatitis C virus infection

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Background: Hepatitis C virus (HCV) dysregulates innate and adaptive immune responses while monocytes (M) play a crucial role in linking innate and adaptive immunity to control viral infection. A transcription factor T-bet is upregulated to dampen M functions via the c-Jun N-terminal kinase (JNK) pathway, followed by enhanced Tim-3 expression in chronic HCV infection. However, the molecular mechanisms that control the expression in M are yet unknown. miR-155 has been implicated as a key regulator controlling diverse biological processes through posttranscriptional repression, but the influences of miR-155 on these regulators and effectors still need to be studied.

Methods: Forty HCV-infected patients and 40 healthy subjects (HS) were recruited, THP-1 cells (human acute monocyte leukemia cell line) were cultured with HCV-infected Huh 7.5 cells. The expression levels of miR-155 and *JNK1/JNK2/JNK3* were measured by real-time RT-PCR. IL-10/IL-12 was detected by flow cytometry. THP-1 cells were transfected with mimics-155 and negative control, SOCS1, p-STAT1, p65, p-smad, p-p38, and p-JNK were measured by Western blot. TNF-α levels were measured by ELISA. Student's *t*-test was used in statistics.

Results: The study showed that miR-155 was upregulated in $CD14^+$ M in HCV-infected patients compared to healthy subjects ($P<0.05$). Moreover, the upregulation of miR-155 in $CD14^+$ M from HCV-infected patients induced TNF-α production and *JNK* gene expression, which, in turn, led to T-bet upregulation. Also, miR-155 upregulation in $CD14^+$ M of HCV-infected patients increased the IL-12 and decreased the IL-10 production.

Conclusions: The obtained results indicated that miR-155 upregulation in M during HCV infection enhances the activation of TNF-α and JNK pathways, promotes the expression of transcription factor T-bet, and triggers pro- and anti-inflammatory mediators. Together, these data reveal new information regarding the mechanisms of chronic HCV infection.

Keywords: Hepatitis C virus (HCV); monocyte (M); microRNA-155 (miR-155); T-bet; immune regulation

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Introduction

Hepatitis C virus (HCV) is a blood-borne, single-stranded, positive-sense RNA virus. Approximately 70–80% of individuals infected with HCV present chronic infection, leading to cirrhosis and hepatocellular carcinoma (HCC) (1). Over time, HCV has evolved numerous strategies to evade host immunity and harness virus persistence (2). Moreover, prolonged immune cell activation during chronic infection drives viral replication and disease progression (3).

In chronic HCV infection, the functionality of both innate and adaptive immunity is affected. Monocytes are bone marrow-derived phagocytes involved in critical immune processes that convert innate immunity to adaptive to defend the host against pathogens (4,5). Our previous research study demonstrated that HCV inhibits IL-12 production and STAT-1 phosphorylation (p-STAT-1) in monocyte (M) through crosstalk between Tim-3 and PD-1 and SOCS-1 (6). Tim-3 is upregulated following Toll-like receptor (TLR) stimulation, and associated with the downregulation of IL-12 and IL-10 (7). T-bet is a Th1-specific transcription factor that represses Th2 cytokine expression and directly activates IFN-γ activity (8). Interestingly, T-bet is expressed in DCs, and it directly regulates inflammatory arthritis. In the absence of T-bet, DCs are unable to efficiently prime Th1 cell responses (9). Reportedly, T-bet is induced by HCV core/gC1qR interaction via the c-Jun N-terminal kinase (JNK) pathway, followed by enhanced Tim-3 expression, leading to dampened M function during chronic HCV infection (10). However, the molecular mechanisms that control its expression in M are largely unknown.

Mammalian microRNAs (miR) are ~22 nucleotide noncoding RNA oligonucleotides highly conserved during evolution and regulate gene expression at a posttranscriptional level through targeting mRNAs (11). miR has been strongly associated with cancer. Moreover, previous studies reported on a correlation between inflammation, innate immunity, and microRNAs expression. Emerging evidence supported that miR-155, miR-146, and miR-21 are relevant to monocyte-derived macrophages inflammatory activation and affect innate immunity (12). However, the lineage of liver resident macrophages and liver-infiltrating blood monocytes differentiating in macrophages are different, and the character of miR-155 in $CD14^+$ M needs to be further explored. Bala *et al.* found an increased expression of miR-155 and miR-122 in the serum of HCV-infected patients. They also suggested that increased miR-155 stimulates TNF-α production in chronic HCV infection (13). A few years later, Cheng *et al.* discovered that HCV-induced miR-155-regulated Tim-3 expression enhances IFN-γ production in natural killer (NK) cells by improving signal transducer and activator of transcription 5 (STAT-5) phosphorylation (14). However, the precise mechanism underlying miR-155 induction in monocytes during HCV infection remains to be elucidated.

In this study, we examined miR-155 and T-bet expression in $CD14^+$ M, and assessed their effect on pro- and anti-inflammatory cytokine production in individuals with chronic HCV infection. We discovered that miR-155 was upregulated in $CD14^+$ M and THP-1 post-stimulation by TLR4 agonist, HCV core, and NS5 proteins or incubation with HCV^+ Huh7.5 cells. Furthermore, miR-155 upregulation in $CD14^+$ M during HCV infection enhanced the activation of TNF-α and JNK pathways, promoted the expression of transcription factor T-bet, and triggered pro- as well as anti-inflammatory mediators: IL-12, TNF-α, and IL-10.

We present the following article in accordance with the STROBE reporting checklist (available at https://dx.doi.org/10.21037/atm-21-2620).

Methods

Subjects

In this study, 40 HCV-infected patients,40 healthy subjects (HS) and 12 HCV-infected patients who achieved sustained virological response (SVR) were recruited from Tangdu Hospital (Shaanxi, China). All the enrolled patients were virologically and serologically positive for HCV before antiviral treatment (*Table 1*). We excluded patients who had liver cirrhosis diagnosed by B ultrasound; FibroScan showed their liver stiffness was F1 to F2. The direct antiviral drugs (DAA) used for HCV-infected patients were Sofosbuvir and Velpatasvir tablets (Epclusa) for 12 weeks with or without ribavirin according to patients' HCV genotypes. The 40 HSs were negative for HBV, HCV, and HIV infection. This study was approved by the Institutional Review Boards (IRB) of the Fourth Military Medical University (TDLL-2012029). All subjects signed the consent form before participation in the study. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

$CD14^+$ monocytes isolation and culture

Human peripheral blood mononuclear cells (PBMCs) were

Table 1 Characteristics of participants in this study

Group	Number	Age (years), mean (range)	Sex (male/female)	HCV-RNA (10^6 IU/mL), mean (range)	HCV genotype	Liver status	ALT (U/L)
Patients with chronic HCV infection	40	50 (19 to 64)	23/17	18.04 (0.0269–58.9)	1b (n=25), 2a (n=13), 3a (n=2)	Non-cirrhotic FibroScan F1-F2	61.41±102.78
Healthy participants	40	36 (23 to 45)	7/33	N/A	N/A	Normal	22.52±11.64
Patients with SVR	12	46 (19 to 58)	9/3	N/A	1b (n=8), 2a (n=4)	Non-cirrhotic FibroScan F1-F2	38.25±19.85

N/A, not applicable; ALT, alanine transaminase.

isolated using Ficoll-Percoll gradient (Sigma-Aldrich, St. Louis, MO, USA). $CD14^+$ monocytes were further isolated from PBMCs using anti-CD14 magnetic beads, according to the manufacturer's instructions (MiltenyiBiotec, Auburn, CA, USA). The purity of monocytes was detected by flow cytometry. The cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA) in a humidified atmosphere containing 5% CO_2/95% air at 37 ℃.

THP-1 cell culture and stimulation

THP-1 cells (human acute monocyte leukemia cell line, American Type Culture Collection, Maryland, MD, USA) were cultured in RPMI-1640 supplemented with 10% FBS. Lipopolysaccharide (LPS) was purchased from Sigma-Aldrich. HCV core and NS5 recombinant proteins were purchased from BioDesign (USA). In some experiments, the cells were incubated with or without 100 ng/mL LPS for 6 h (13,15,16). An equivalent of 2 μg/mL HCV core or NS5 proteins was used to stimulate THP-1 cells (13). Then, total RNA was isolated and estimated by PCR analysis, as described below.

Co-culture of monocytic THP-1 cells with HCV-infected Huh 7.5 cells

Plasmid HCV J6/JFH-1(HCV Japanese fulminant hepatitis-1) was isolated from *E. coli* by a plasmid miniprep kit (Tiangen Co., China) and linearized with Xba I (Thermo Scientific, Rockford, IL, USA). Transcript Aid T7 high-yield transcription kit (Thermo Scientific) was used to transcribe RNA. Huh7.5 cells were transfected with HCV RNA in a 24-well plate using DMRIE-C reagent (Invivogen, San Diego, CA, USA) at a density of 5×10^5 cells/well in a medium without antibiotics. HCV core protein was observed 72 h after transfection using a fluorescence microscope as previously described (7,10,17). HCV RNA was quantified by quantitative PCR from the supernatants of cell culture (Qiagen, Hilden, Germany). Suspended THP-1 cells were co-cultured with HCV-transfected adherent Huh7.5 cells and without HCV-transfected adherent Huh7.5 cells in RPMI media. The cells were collected after 6 h, and THP-1 RNA was isolated; then, *miR-155* expression was detected by real-time PCR as described below.

Quantifying miR-155 by RT-PCR

Total RNA was isolated from monocytes using TRIzol reagent (Invitrogen, CA, USA), according to the manufacturer's instructions. Specific primers for *miR-155* and *U6* miRNA were obtained from Applied Biosystems (Grand Island, NY, USA). The expression levels of miRNAs were measured by real-time RT-PCR using specific miRNA assay primer sets and TaqMan Universal Master Mix (Applied Biosystems). Quantitative RT-PCR was carried out on the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, California, USA); the miR-155 level was normalized to *snRU6* levels and expressed as fold-changes using the $2^{-\Delta\Delta Ct}$ method.

Monocyte transfections and flow cytometry

To transiently upregulate or block miR-155 expression, miR-155 mimics, miR-155 inhibitor, and negative controls were synthesized from GenePharma Co., Ltd (Shanghai, China) and transfected into human monocytes. First, 1×10^6 human$CD14^+$ monocytes from HCV-infected patients were transfected with 100 pmol miR-155 inhibitors or the negative control, using the HiPerFect Transfection Reagent (Qiagen, Germany). Then, 1×10^6 human $CD14^+$ monocytes from health control were transfected with

100 pmol miR-155 mimics or the negative control. The transfection efficiency was assessed by flow cytometry. After 6 h transfection, the cells were subjected to centrifugation at 1,500 rpm for 5 min at room temperature and cultured in RPMI 1640 medium containing 10% FBS for 24 h. After transfection, monocytes were used to detect T-bet expression, and intracellular expression of IL-10/IL-12 production by flow cytometry. Specifically, monocytes were fixed and permeabilized with Cytofix/Cytoperm™ Plus (BD Biosciences, Franklin Lakes, NJ, USA), followed by incubation with PE-conjugated anti-T-bet antibody and stained with FITC-conjugated anti-CD14 antibody (eBioscience, San Diego, CA, USA). Isotype controls (eBioscience) were used to determine the level of background staining. $CD14^+$ monocytes were mixed with 5 μg/mL Brefeldin A (BioLegend, San Diego, CA, USA) 5 h before harvesting to forbid cytokine secretion. $CD14^+$ monocytes were fixed and permeabilized with BD Cytofix/Cytoperm™ Plus (BD Biosciences), followed by incubation with anti-IL-10 and IL-12 antibodies.

Analysis for JNK expression

$CD14^+$ monocytes from HCV-infected patients were transfected with 100 pmol miR-155 inhibitor or negative control, and HS were transfected with 100 pmol miR-155 mimics or the negative control as described above. Total RNA was isolated from monocytes using TRIzol reagent (Invitrogen), according to the manufacturer's protocol. The reverse transcription of 1 μg total RNA was performed using the PrimeScript™ reverse transcript (RT) reagent kit (TaKaRa Biotechnology, Dalian, China). Each assay was performed in triplicate and normalized to *GAPDH* expression. The primer sequences for *JNK1/JNK2/JNK3* and *GAPDH* were synthesized by Shanghai Sangon Co., Ltd (Shanghai, China). Relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method.

Western blot

THP-1 cells were transfected with 100 pmol mimics-155 and negative control. The cells were lysed on ice in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, 0.5% deoxycholic acid, 0.1% SDS, 1% Triton X-100). Proteins were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes. Then, membranes were blocked with 5% milk-0.5% Tween-20 in Tris-buffered saline and probed with the anti-SOCS1 and anti-p-STAT1 (Cell Signaling Technology (CST), Shanghai, China) at 4 ℃ overnight. Subsequently, the membrane was incubated with horseradish peroxide-conjugated secondary antibody (CST). The immunoreactive bands were detected using an enhanced chemiluminescence assay kit (Beyotime Biotechnology, Shanghai, China).

THP-1 cells were divided into four groups: the control group incubated in PBS, and the experimental group treated with LPS (Sigma-Aldrich, MO), HCV CORE, or HCV NS5 (Abcam, Shanghai, China) antigens, respectively. After 1 day of culturing, the cells were lysed and protein levels were assessed by Western blot. The changes in cell signaling were detected after the stimulation of each antigen, and the phosphorylation of p65, smad, p38, and JNK proteins was detected. The applied antibodies were p65, p-smad, p-p38, and p-JNK (Abcam).

Cytokine measurements

TNF-α levels were measured in the culture supernatants of HCV-infected patients and HS monocytes treated with miR-155 inhibitor or mimics-155 and the corresponding negative controls as described above, followed by measuring cytokine production using commercially available enzyme-linked immunosorbent assay (ELISA) kits (ExCell Bio, Taicang, China) according to the manufacturer's instructions.

Statistical analysis

The data were analyzed using GraphPad Prism version 5 software. Student's *t*-test or Mann-Whitney U test with one-tailed method were used to determine the statistical significance of the differences between groups. A P value<0.05 indicated statistical significance.

Results

miR-155 was upregulated in monocytes from chronically HCV-infected individuals

miR-155 is upregulated in various immune cells in HCV-infected individuals (12,13). To analyze the expression level of miR-155 in monocytes during HCV infection, $CD14^+$ monocytes from PBMCs of 12 chronically HCV-infected patients and 12 HS were isolated, and the miRNA expression was examined using real-time RT-PCR. As shown in *Figure 1A*, >90% $CD14^+$ monocytes were isolated from PBMCs. Chronically HCV-infected individuals exhibited a significantly higher level of miR-155 expression in $CD14^+$

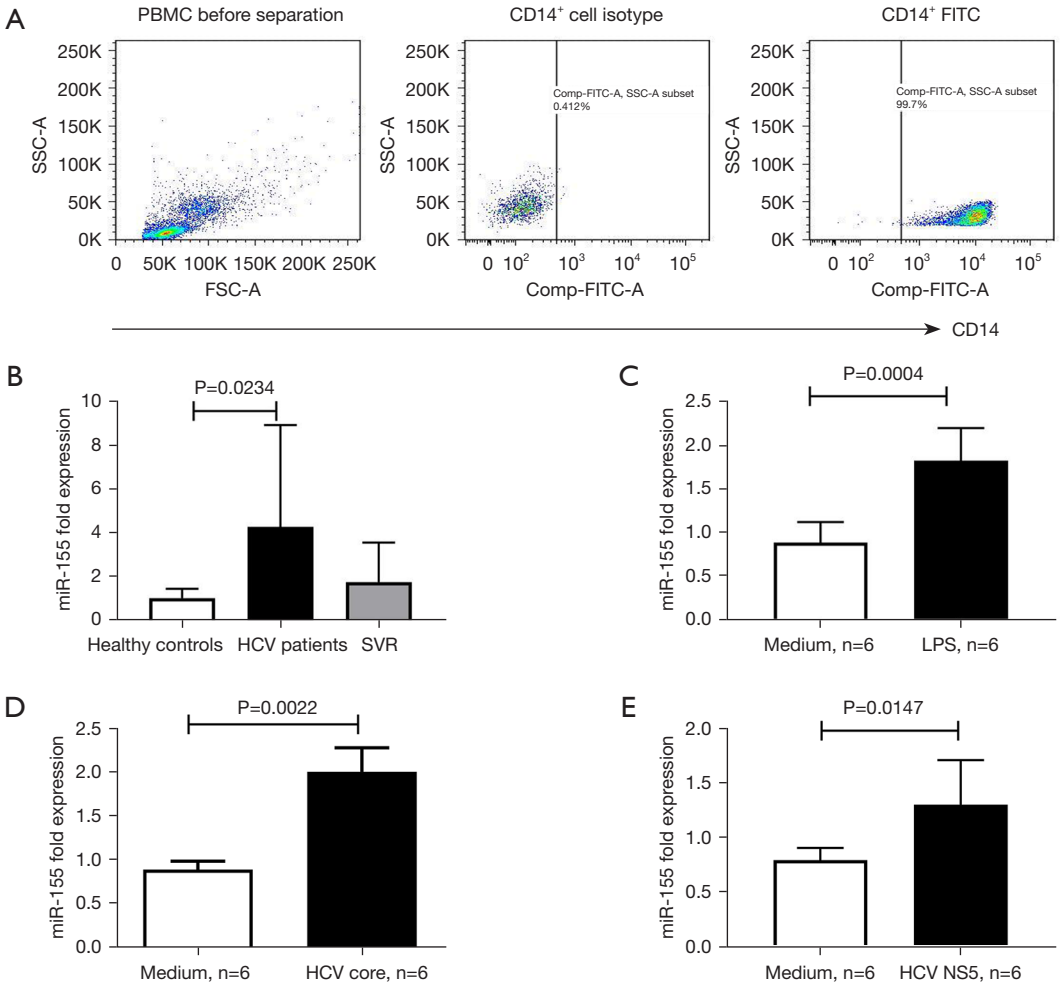

Figure 1 miR-155 upregulation in monocytes of chronically HCV-infected individuals. (A) Representative dot plots of flow cytometric analysis for CD14$^+$ monocytes in PBMCs, stained with isotype and CD14-FITC antibodies, >90% CD14$^+$ monocytes were isolated from PBMCs. (B) CD14$^+$ monocytes were purified from PBMCs of 12 healthy controls, 12 HCV-infected patients and 12 HCV-infected patients who achieved sustained virological response (SVR), after which miR-155 levels were examined by real-time RT-PCR. miR-155 fold-change ($2^{-\Delta\Delta Ct}$) from HCV-infected patients was normalized to that of healthy controls. (C-E) Monocytic THP-1 cells were stimulated with or without LPS, HCV core, or HCV NS5 for 6 h, followed by real-time RT-PCR analysis of *miR-155* levels (n=6, repeated three times).

monocytes with a more than two-fold increase compared to healthy controls (P<0.05). Interestingly, after treatment, miR-155 expression in patients who achieved sustained virological response (SVR) did not return to normal levels, although it showed a decreased trend compared to that before treatment (*Figure 1B*). Recent studies demonstrated that the increased miR-155 expression in PBMCs of chronic HCV-infected patients could be induced by TLR stimulation. Consequently, we stimulated THP-1 cells with TLR4 ligand-LPS, HCV core, and NS5. As shown in *Figure 1C*, an increase in miR-155 levels was observed in THP-1 monocytes following 6 h LPS stimulation. Similar results were observed in miR-155 induction by HCV core or NS5 compared to the control medium alone (*Figure 1D,1E*). These findings illustrated that HCV upregulates miR-155 expression in multiple contexts.

miR-155 was upregulated in THP-1 cells co-cultured with HCV Huh7.5 cells*

To further evaluate the miR-155 expression after HCV infection, we exploited an HCV culture system to simulate

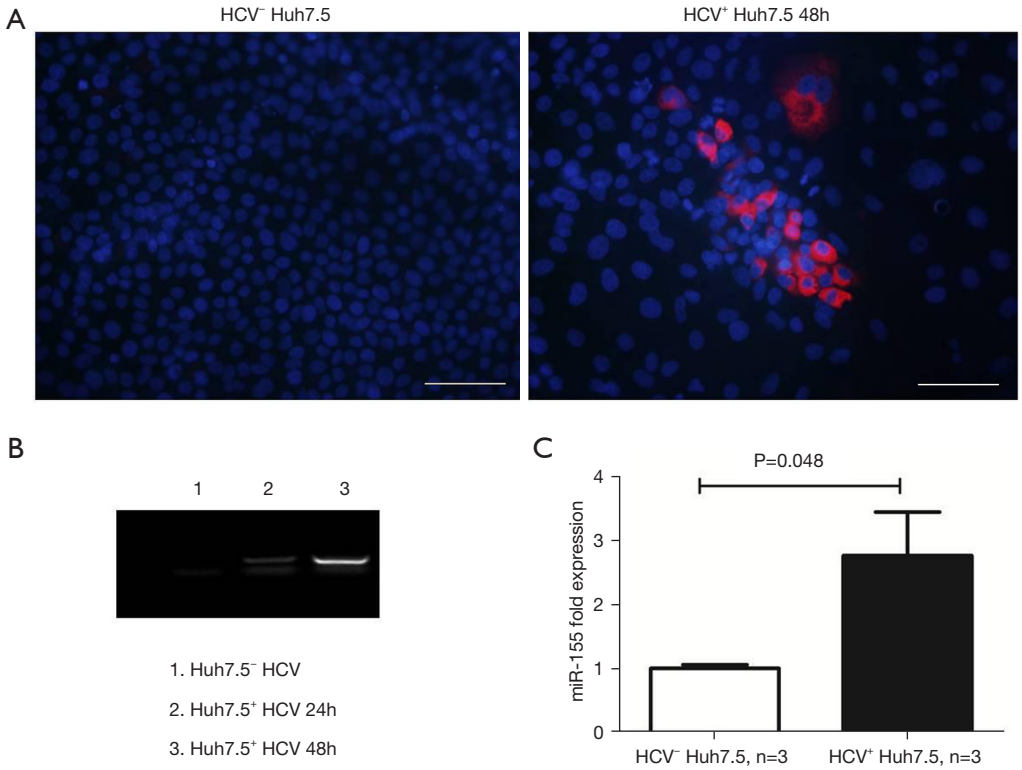

Figure 2 miR-155 up-regulation in THP-1 cells co-cultured with HCV$^{+/-}$ Huh7.5 cells. THP-1 cells were co-cultured with HCV^{+} or HCV^{-} Huh7.5 cells, followed by real-time RT-PCR analysis of *miR-155* expression. (A) Huh7.5 cells were transfected with JFH-1 or control (HCV^{-} Huh7.5). HCV core protein (red) was detected by immunofluorescence staining after 48 h, and Hoechst was used to visualize the nuclei. Scale bar represents 50 μm. (B) HCV RNA from the culture supernatant was detected 24 h after transfection. (C) Total RNA was isolated after 24 h of transfection and subjected to TaqMan miRNA assay for miR-155.

the viral infection *in vivo*. Purified JFH-1 plasmids were transfected into Huh7.5 cells. After 48 h, the supernatant of the medium was collected to assess the HCV viral load. HCV core protein was detected by immunofluorescence after 48 h incubation (*Figure 2A*). HCV RNA content was measured at 8.9×10^{8}–4.8×10^{9} IU/mL in the supernatant of the medium by RT-PCR and agarose gel electrophoresis 24 h after transfection (*Figure 2B*). THP-1 cells were then co-cultured with HCV^{+}/HCV^{-} Huh7.5 cells for 6 h. As shown in *Figure 2C*, miR-155 was significantly upregulated in THP-1 cells incubated with HCV^{+} Huh7.5 cells compared to the negative controls. These data suggested that HCV induces miR-155 expression in monocytic THP-1 cells.

miR-155 induces TNF-α production during chronic HCV infection

TNF-α is a pro-inflammatory cytokine that has a key role in immune regulation, including programmed cell death and inflammation (15,16). miR-155 is strongly induced by poly(I:C) and IFN-α in macrophages, and the JNK pathway is involved in the upregulation of miR-155 expression in response to poly(I:C) or TNF-α (18). Also, the enforced expression of miR-155 in CD14^{+} monocytes from rheumatoid arthritis and healthy subjects stimulates the production of TNF-α (19). To further assess whether HCV-induced miR-155 controls TNF-α expression in monocytes, we transfected miR-155 mimics and inhibitors in monocytes from healthy subjects (HS) and HCV patients, respectively. The efficacy of transfection is shown in *Figure 3A*. The expression of TNF-α was detected by ELISA 24 h after transfection (*Figure 3B*). Compared to the negative control, TNF-α was upregulated in HS monocytes transfected with miR-155 mimics while it was decreased in HCV monocytes transfected with miR-155 inhibitor, albeit not significantly (P>0.05).

Several JNK molecules were detected by RT-PCR (*Figure 3C*). JNK1 was upregulated in HS monocytes transfected with miR-155 mimics and reduced in HCV

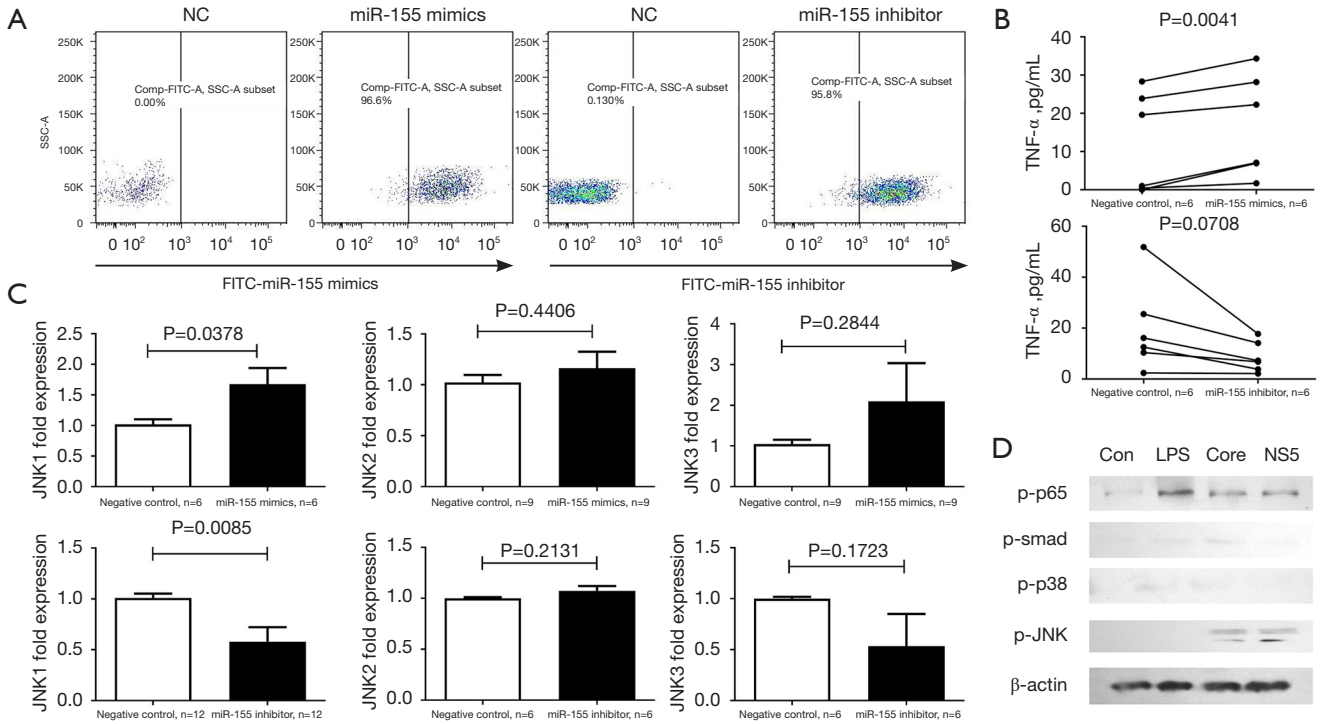

Figure 3 miR-155 induces TNF-**α** production and activates JNK signaling during chronic HCV infection. CD14$^+$ monocytes isolated from chronically HCV-infected individuals were transfected with miR-155 inhibitors or negative control while CD14$^+$ monocytes isolated from HS were transfected with miR-155 mimics or negative control for 24 h. TNF-α and JNK1, 2, 3 were detected by ELISA and RT-PCR. (A) Transfection efficiency was measured by flow cytometry after 6 h transfection of CD14$^+$ monocytes with fluorescent (FAM) miR-155 mimics and fluorescent (FAM) miR-155 inhibitors. (B) TNF-α secreted in the culture media was measured by ELISA. (C) After CD14$^+$ monocytes were transfected with miR-155 mimics or miR-155 inhibitor, the expression of *JNK1* (n=6 or 12), *JNK2* (n=9 or 6), and *JNK3* (n=9 or 6) was determined by RT-PCR. (D) HCV core and NS5 antigens promote the activation of JNK signaling, including JNK and p65 in THP-1 cells.

monocytes transfected with miR-155 inhibition (n=12, P<0.05). No significant difference was detected in JNK2 and JNK3 expression (n=12, P>0.05). These data suggested that miR-155 might regulate JNK1 and TNF-α expression in monocytes following HCV infection. As shown in *Figure 3D*, HCV core and NS5 antigens activated the JNK signals, although the changes in these signals were small when stimulated by LPS, while smad and p38 were not significantly affected by each signal stimulation. Since JNK signaling is the main pathway of regulating miR-155, and HCV-elevated miR-155 might be related to the activation of JNK signaling by HCV antigen.

miR-155 upregulates T-bet expression in monocytes from chronically HCV-infected individuals

In a previous study, we showed that T-bet was upregulated and involved in inhibiting monocytes' functions during HCV infection, and HCV-induced T-bet upregulation and IL-12 inhibition in monocytes were regulated by the JNK signaling pathway (10). As shown in *Figure 3C*, JNK1 was upregulated in HS monocytes transfected with miR-155 mimics, while it was decreased in HCV monocytes transfected with miR-155 inhibitor. To further evaluate whether HCV-induced miR-155 controls T-bet production in monocytes, PBMCs from HCV-infected patients and HS were isolated and transfected with either miR-155 inhibitors or miR-155 mimics, and T-bet was detected by flow cytometry. As shown in *Figure 4A*,*4B*, the expression of T-bet in monocytes from HS was significantly increased after 24 h transfection with mimics of miR-155 compared to those treated with the negative control (n=9, P=0.0015). Moreover, T-bet was decreased after inhibiting miR-155 24 h post-transfection

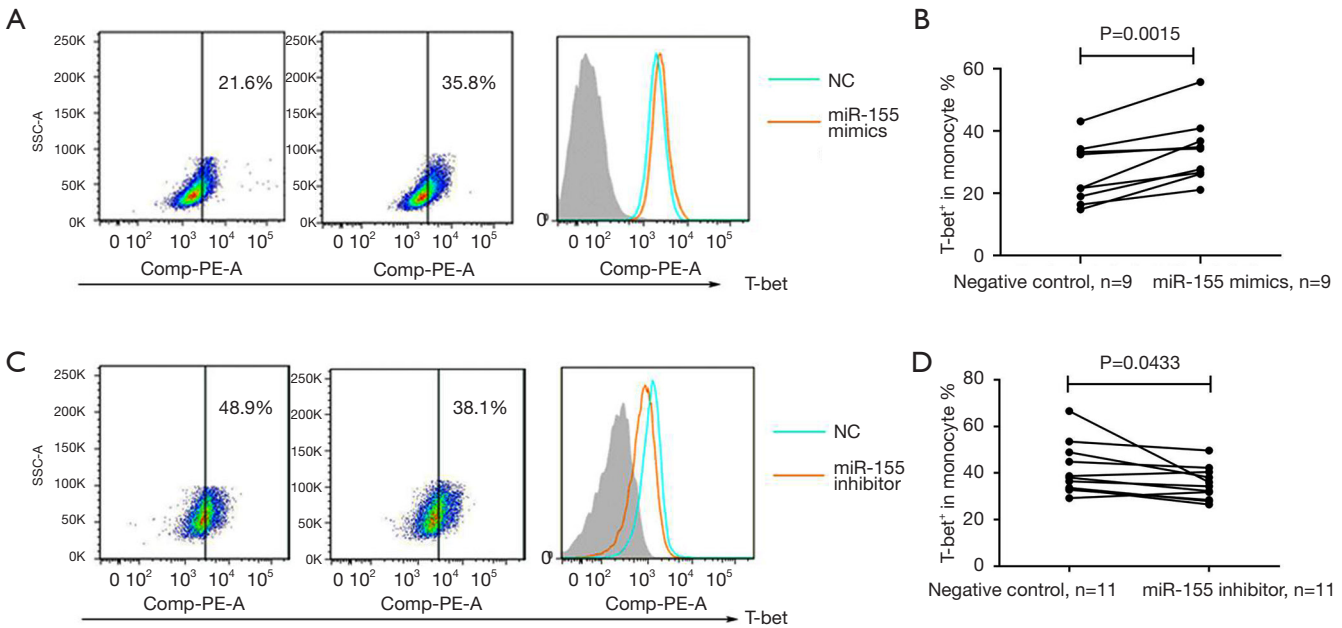

Figure 4 miR-155 upregulates T-bet expression in monocytes of chronically HCV-infected individuals. (A) $CD14^+$ monocytes of HS were transfected with miR-155 mimics, and T-bet was detected by flow cytometry 24 h after transfection. (B) The expression of T-bet in monocytes from HS was increased after 24 h transfection (n=9, P=0.0015). (C) $CD14^+$ monocytes of HCV-infected individuals were transfected with miR-155 inhibitor, and T-bet was detected by flow cytometry 24 h after transfection. (D) T-bet in monocytes from HCV was decreased after inhibiting miR-155 (n=11, P=0.0433).

(*Figure 4C,4D*) (n=11, P=0.0433). Collectively, these data suggested that HCV-induced miR-155 induction in monocytes promotes T-bet expression.

miR-155 regulates inflammatory cytokine IL-12 expression in monocytes

IL-12p70 produced by monocytes and DCs have a critical role in the development of IFN-γ producing T cells and NK-cell activation (13). We investigated whether miR-155 regulates IL-12 production in monocytes after HCV infection. $CD14^+$ monocytes were further isolated from PBMCs using anti-CD14 magnetic beads according to the manufacturer's instructions. miR-155 mimic was transfected in HS monocytes, and miR-155 inhibitor was transfected in HCV monocytes. After 24 h, IL-12 was significantly upregulated in HS monocytes transfected with miR-155 mimic (*Figure 5A*) (n=5, P=0.0009), while it was markedly reduced in HCV monocytes transfected with miR-155 inhibitor (*Figure 5B*) (n=5, P=0.005). Together, these data demonstrated that miR-155 regulates monocyte cytokine IL-12 production during HCV infection.

miR-155 regulates inflammatory cytokine IL-10 expression in monocytes

In addition to IL-12, IL-10 are cytokines with potent anti-inflammatory properties that have a central role in limiting host immune response to pathogens, preventing damage to the host, and maintaining normal tissue homeostasis. Therefore, we investigated miR-155-mediated regulation of IL-10 expression in monocytes of HCV patients and HS (14). $CD14^+$ monocytes were isolated from PBMCs of HCV-infected patients and HS. miR-155 mimic was transfected in HS monocytes, and miR-155 inhibitor was transfected in HCV monocytes. After 24 h incubation, IL-10 was significantly decreased in HS monocytes transfected with miR-155 mimic (*Figure 6A*) (n=6, P=0.0143) and increased in HCV monocytes transfected with miR-155 inhibitor (*Figure 6B*) (n=5, P=0.0039). These data showed that in addition to IL-12, anti-inflammatory cytokine IL-10 production in monocytes was also affected by miR-155 during chronic HCV infection.

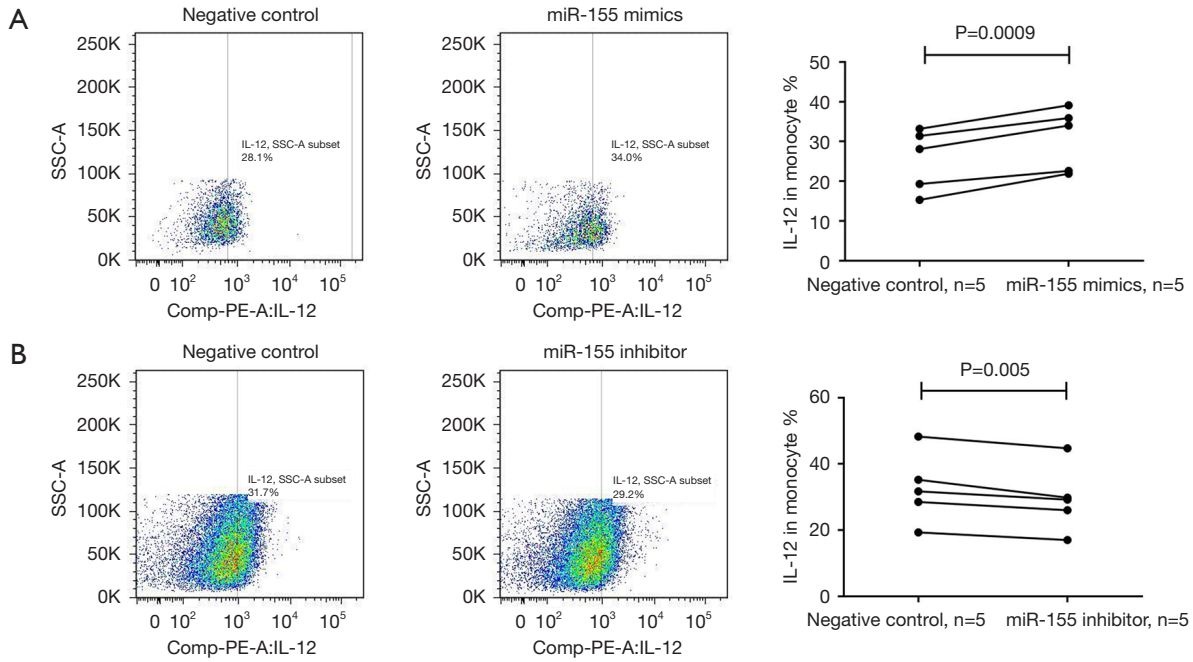

Figure 5 miR-155 regulates inflammatory cytokine IL-12 expression in monocytes. The intracellular expression of IL-12 was detected in HS CD14$^+$ monocytes transfected with miR-155 mimics (A) (n=5, P=0.0009) and CD14$^+$ monocytes of HCV-infected individuals transfected with miR-155 inhibitor (B) (n=5, P=0.005) by flow cytometry 24 h after transfection.

miR-155 regulates inflammatory cytokine expression in monocytes through the SOCS1 signaling pathway

Reportedly, the expression of IL-12p70 and IL-10 can be regulated through SOCS1 and STAT1 (15,16,20). In a previous study, we showed that silencing *SOCS1* gene expression decreased Tim-3 expression and improved IL-12 production in THP-1 cells (18). The base pairing to the 3'-untranslated regions (UTRs) of mRNA facilitates miRNAs to function by repressing their target genes at the posttranscriptional level. Furthermore, miR-21 and miR-155 target and downregulate *SOCS1* and *SOCS6* gene expression (19). We also characterized the correlation between miR-155 and IL-12/IL-10/SOCS1 in monocytes of HCV-infected patients. An analysis by TargetScan identified the 3'-UTRs of SOCS1-miR-155 binding sites (*Figure 7A*). Therefore, miR-155 mimic was transfected in HS monocytes. After 24 h, the expression of *SOCS1* mRNA was decreased in HS monocytes transfected with miR-155 mimic (*Figure 7B*, n=3, P=0.0035). Also, SOCS1 expression showed a declining trend after mir-155 mimic transfection, but no significant change was detected in protein level, while p-STAT1 was barely affected (*Figure 7C*,*7D*).

Discussion

During the early stage of HCV infection, innate immune cells recognize pathogen-associated molecular patterns (PAMP) via pattern recognition receptors (PRRs). These TLRs are PRRs that recognize a wide range of PAMPs and are expressed in Kupffer cells, monocytes, DCs, hepatocytes, hepatic stellate cells, and other cell types (21). The monocytes function as patrolling cells and the primary mediators of the inflammatory response during HCV infection (22). However, HCV viral proteins can interact with monocyte and disrupt Toll-interleukin 1 receptor (TIR) signaling (23). TLRs signal stimulates pro-inflammatory and antiviral genes through adapter molecules, including myeloid differentiation factor 88 (MyD88) and TIR domain-containing adaptor-inducing IFNb (Trif). MyD88-induced signals activate NF-κB, IRF-7, and JNK, while Trif-dependent signals activate NF-κB and IRF-3 (24), leading to immune-response gene activation. However, HCV-based strategies avoid the activation of antiviral pathways by TLRs and their ligands. Intriguingly, HCV impairs innate immune pathways that limit HCV replication via type I IFNs while generating a chronic inflammatory state that causes persistent liver injury (21).

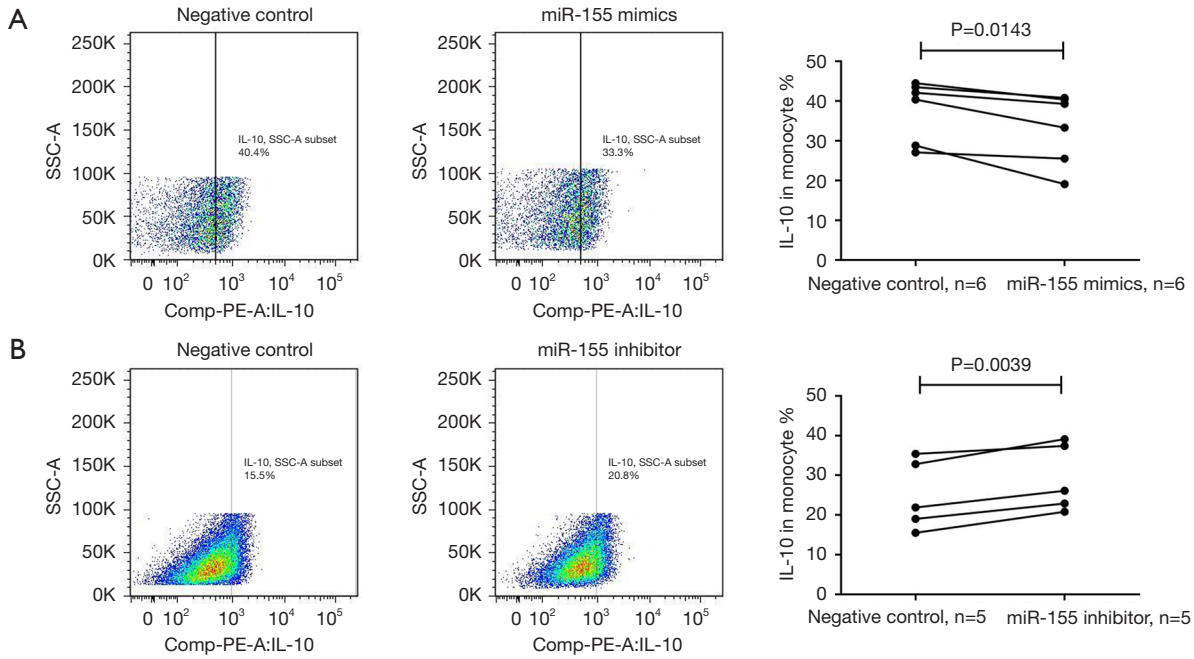

Figure 6 miR-155 regulates inflammatory cytokine IL-10 expression in monocytes. (A) The intracellular expression of IL-10 in $CD14^+$ monocytes of HS was detected by flow cytometry 24 h after transfection with miR-155 mimics, the IL-10 expression decreased after miR-155 increased (n=6, P=0.0143). (B) The intracellular expression of IL-10 in $CD14^+$ monocytes of HCV-infected individuals was detected 24 h after transfection with miR-155 inhibitor, the IL-10 expression increased after miR-155 was inhibited (n=5, P=0.0039).

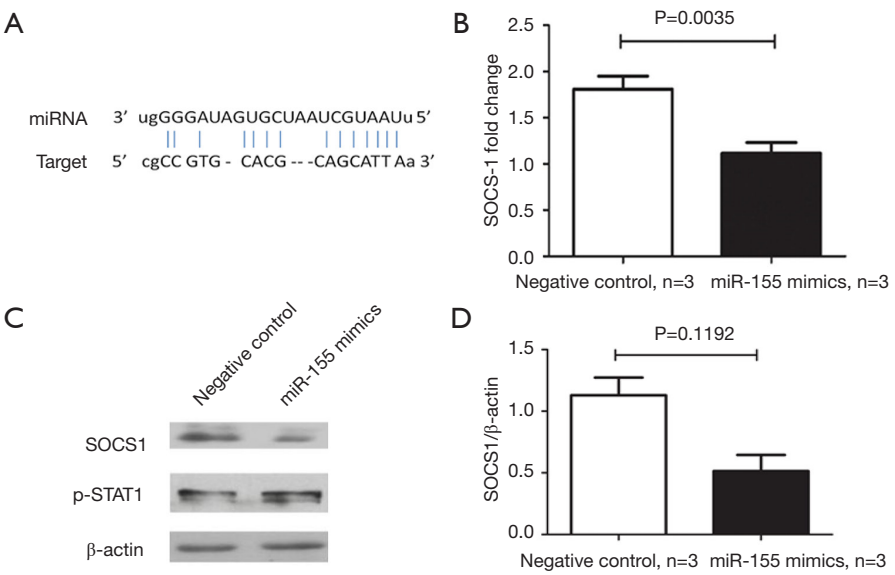

Figure 7 miR-155 regulates inflammatory cytokine expression in monocytes through SOCS1. (A) The predicted binding sites of miR-155 in the 3'-UTRs of SOCS1. (B) The expression of *SOCS1* mRNA was detected by RT-PCR (n=3, P=0.0035). (C,D) HS $CD14^+$ monocytes were transfected with miR-155 mimics; SOCS1 and p-STAT1 were detected by WB 24 h after incubation; and the gray values were measured.

MicroRNAs are small noncoding RNAs that target mRNA through specific base-pair interactions between the 5'-end of miRNA and UTRs, especially the 3'-UTR of mRNAs. In addition, miRNAs can inhibit the target gene expression by either mRNA degradation or translational repression (25). Increasing evidence showed that miR-155 expression is elevated in PBMCs of chronically HCV-infected patients but not in individuals who undergo successful antiviral therapy (26). Herein, we found that miR-155 expression in $CD14^+$ monocytes of chronic HCV patients is significantly upregulated compared to healthy subjects (HS) and has a pivotal role in controlling cellular functions during HCV infection. The upregulation of miR-155 was also found in THP-1 cells stimulated with HCV core, NS5, or LPS. In addition, the HCV cell co-culture system demonstrated that miR-155 expression in THP-1 cells could be induced by HCV^+ Huh7.5 *in vitro*.

Due to the limited cells from HCV patients and healthy donors, THP-1 cells were used in the experiment instead of the monocytes from human bodies in some experiments. THP-1 cells are widely used in monocyte and macrophage studies. They can imitate the monocyte as well as its differentiation, when encountering immune stimulation. THP-1 cell can be differentiated to M0 macrophages by PMA; then M0 macrophages can be polarized into M1 with human IFN-γ and LPS, and into M2 with human IL-4 and IL-13 as described before (27). Two polarized states of macrophages (M1 and M2) have important roles in pro-inflammatory and anti-inflammatory effects respectively. According to one recent study, the mRNA and protein expression were strongly up-regulated in M1 macrophages compared to M2 macrophages (28). Our results may be inconsistent concerning to the phenotype of THP-1 cells because we used LPS to stimulate THP-1 cells as control. Yet, the cell line experiment could only partially reflect the miR-155 influences, especially when taking cell polarization and liver status into account.

miR-132, miR-146, and miR-155 can be upregulated upon LPS stimulation in human THP-1 monocytes, with miR-146 regulated by NF-κB and miR-155 by JNK (29). TNF-α is a pro-inflammatory cytokine involved in various physiological processes, including inflammation, proliferation, and programmed cell death (20). TNF-α interacts with TNF-R1, recruits TRADD, TRAF2, and RIP, and forms complex I, activating the JNK MAPK signaling pathway (26). The current study demonstrated that miR-155 upregulation in HS could increase JNK1 and TNF-α levels while its inhibition in HCV-infected subjects could reverse this process (decrease JNK1 and TNF-α levels), thus suggesting that HCV upregulates miR-155 expression, induces TNF-α secretion, and activates JNK signaling pathway.

T-bet is a Th1-specific transcription factor of the T-box family that induces the production of IFN-γ. It also promotes the differentiation of Th0 to Th1 cells, stimulating theTh1 immune response and inhibiting the differentiation of Th0 to Th2 cells (30). Our previous data revealed that T-bet expression is significantly upregulated in $CD14^+$ monocytes derived from chronic HCV patients and is induced by HCV core/gC1qR interaction via the JNK signaling pathway. Moreover, HCV core-induced upregulation of T-bet and Tim-3 expression in M/M_Φ can be abrogated by incubation with SP600125-an inhibitor for the JNK signaling pathway (10). In this study, based on the observation that miR-155 activates TNF-α and JNK signaling pathways, we proposed that miR-155 affects T-bet and JNK expression. To test this hypothesis, we manipulated miR-155 expression in monocytes and detected T-bet by flow cytometry. The displayed data enforced the hypothesis that expression of miR-155 in HS monocytes promotes T-bet production, which in turn suggests a critical role of miR-155 in controlling T-bet production and JNK expression in human monocytes, although we did not prove the direct link of the expression of JNK to T-bet. Thus, the mechanism of the regulation associated with transcriptional factors needs to be further explored.

IL-12 is a 70–75 kDa heterodimeric cytokine composed of a 40-kDa H chain and a 35-kDa L chain. It is mainly produced by activated monocytes, as well as myeloid DCs (mDCs) (13). Microbial gene products also upregulate costimulatory or inhibitory receptors on monocytes or mDCs and modulate the secretion of IL-12, having an essential role in the transition between innate and adaptive immune responses (24). IL-10 suppresses hepatic inflammation and reduces fibrosis scores in chronic hepatitis (9). However, evidence suggested that long-term treatment of HCV patients with IL-10 elevates HCV viremia. IL-12/IL-10 produced by monocytes induces various effects on NK and T cells, having an essential role in the transition between innate and adaptive immune responses and the maintenance of homeostasis. This study showed that IL-10 was augmented and IL-12 was inhibited in monocytes from HCV infected patients treated with miR-155 inhibitor. Simultaneously, IL-10 was inhibited, and IL-12 was augmented in HS monocytes treated with miR-155 mimics. The obtained data also suggested that miR-155 positively affected the pro-inflammatory innate response in monocyte by subverting the inhibitory effect of IL-10

and upregulating IL-12 expression. During chronic HCV infection, increased miR-155 in monocytes promoted the inflammatory signaling by activating IL-12 production and inhibiting IL-10 expression in order to control HCV virus replication.

Furthermore, computational miRNA target prediction algorithms identified multiple potential targets of miR-155. Thus, miR-155, like many other miRNAs, may target a wide spectrum of genes, such as *SOCS1*, which could be involved in the regulation of multiple independent cells signaling processes (20,31). Our previous data suggested that HCV inhibits immune responses by suppressing the JAK-STAT signaling pathway by regulating SOCS1 and STAT expression (17). SOCS1 was identified as a direct target of miR-155 in $CD14^+$ monocytes, activating JAK/STAT signaling. Monocytes are a major source of inflammatory cytokines, including IL-10 and IL-12. IL-10 has potent anti-inflammatory effects and is essential for the regulation of immune responses. IL-12 production can be limited by SOCS-1, an inducible negative feedback inhibitor of the JAK/STAT signaling pathway (17,32). This study demonstrated that the enhanced IL-12 production and decreased IL-10 production by $CD14^+$ monocytes was regulated via overexpression of miR-155, thus suggesting that miR-155 activates the JAK/STAT pathway. This study identified the expression and function of miR-155 in $CD14^+$ monocytes during chronic HCV infection. miR-155 was induced in CD14+ monocytes by HCV core, NS5, and LPS stimulation, indicating the potential mechanisms for *in vivo* miR-155 induction in patients with chronic HCV infection. Furthermore, miR-155 activates TNF-α and JNK expression, promoted the expression of transcription factor T-bet, and triggered pro- and anti-inflammatory mediators, such as IL-12, TNF-α, and IL-10. The effects of miR-155 on $CD14^+$ monocytes in the process of HCV infection were comprehensively analyzed. However, no direct regulation mechanism between transcription factors and cytokine expression were established, and the specific mechanism of transcription factors regulating cytokine expression need to be further explored.

This study has a few limitations. First, HCV infected patients enrolled in this study were older (age ranging from 19 to 64, mean age of 50 years old) compared to the healthy controls (mean age of 36 years old), which may lead to some bias. Second, we excluded patients with liver cirrhosis confirmed by a B ultrasound, Fibrosan showed their liver stiffness were F1 to F2. We did not compare the patients with chronic hepatitis C to those with liver cirrhosis, considering liver cirrhosis may affect the results. Third, THP-1 cells can differentiate to M0 macrophages, and polarize into M1 or M2 with different stimulations (27). However, the mRNA and protein expression were strongly up-regulated in M1 macrophages compared to M2 macrophages (28). Our results may differ according to the phenotype of THP-1 cells. Fourth, we measured the cytokines at 24 h after transfection instead of at different times and did not examine the kinetics of the production of cytokines in this study.

To sum up, our results indicated that miR-155 upregulation in monocytes during HCV infection enhances the activation of TNF-α and JNK pathways, promotes the expression of the transcription factor T-bet, and triggers pro- and anti-inflammatory mediators, thus potentially balancing immune clearance and injury during chronic HCV infection.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://dx.doi.org/10.21037/atm-21-2620). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the Institutional Review Boards (IRB) of the Fourth Military

Medical University (TDLL-2012029). All subjects signed the consent form before participation in the study.

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