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HCV-induced miR146a controls SOCS1/STAT3 and cytokine expression in monocytes to promote regulatory T cell development

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Abstract

Host innate and adaptive immune responses must be tightly regulated by an intricate balance between positive and negative signals to ensure their appropriate onset and termination while fighting pathogens and avoiding autoimmunity; persistent pathogens may usurp these regulatory machineries to dampen host immune responses for their persistence *in vivo*. Here we demonstrate that miR146a is up-regulated in monocytes from hepatitis C virus (HCV)-infected individuals compared to control subjects. Interestingly, miR146a expression in monocytes without HCV infection increased, whereas its level in monocytes with HCV infection decreased, following Toll-like receptor (TLR) stimulation. This miR146a induction by HCV infection and differential response to TLR stimulation were recapitulated *in vitro* in monocytes co-cultured with hepatocytes with or without HCV infection. Importantly, inhibition of miR146a in monocytes from HCV-infected patients led to a decrease in IL-23, IL-10, and TGF- β expressions through induction of suppressor of cytokine signaling 1 (SOCS1) and inhibition of signal transducer and activator transcription 3 (STAT3), and this subsequently resulted in a decrease in regulatory T cells (Tregs) accumulated during HCV infection. These results suggest that miR146a may regulate SOCS1/STAT3 and cytokine signaling in monocytes, directing T cell differentiation and balancing immune clearance and immune injury during chronic viral infection.

Keywords

HCV; miR146a; monocytes; regulatory T cells; SOCS1; STAT3

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Introduction

Host innate and adaptive immune responses must be tightly regulated by an intricate balance between positive and negative signals to ensure their appropriate onset and termination while fighting pathogens and avoiding autoimmunity; persistent pathogens may usurp these regulatory machineries to dampen host immune responses for their persistence *in vivo*. Hepatitis C virus (HCV), a blood-borne viral infection characterized by a high rate of chronic infection, has evolved multiple strategies to evade host immune responses, thus becoming an excellent model to study the mechanisms of persistent viral infections [1–2]. While the use of direct antiviral agents (DAA) has resulted in a significant improvement in the outcome of HCV treatment, this therapeutic cocktail is still under development and already facing new issues such as viral mutation, relapse, and re-infection following therapy [3–4]. Additionally, the lack of a vaccine for this virus is a major hurdle to control this global infection. The failure to successfully manage this chronic viral infection and to develop an effective vaccine stems from our incomplete understanding of HCV-host interactions that lead to viral persistence.

While a high rate of genetic variability in HCV structural and non-structural proteins (quasispecies) may facilitate viral persistence, HCV-elicited immune responses appear to be too weak to resolve infection in most humans or to protect against re-infection in chimpanzees [5–6]. It appears that virus-mediated impairment of innate and adaptive immune response is a major mechanism by which persistent infection is established [1–2]. We have previously shown that chronically HCV-infected individuals exhibit an aberrant secretion of IL-23 and IL-10 by monocytes, contributing to the differentiation of Th17 cells and accumulation of Foxp3⁺ regulatory T cells (T-regs) [7–10]. However, the precise mechanisms that control IL-23 and IL-10 expression in monocytes and T cell differentiation remain elusive. Notably, aberrant inflammatory activities may also contribute towards immune-mediated injury [11–12]. Therefore, additional studies are required to understand how these immune cells are fine-tuned in host defense and immune injury during chronic viral infections.

microRNAs (miRNAs or miRs) are a class of small, non-coding RNAs that can regulate gene expression through translational repression or target mRNA degradation and have been implicated as negative regulators of innate and adaptive immune responses [13–15]. Genome-wide expression profiling of miRNAs in human monocytes has revealed several endotoxin-responsive miRNAs (miR146a/b, miR155, miR132, and miR125b) that can regulate inflammatory processes at multiple levels [16]. miR146a has been identified as a key modulator of differentiation and function of cells in both innate and adaptive immunity [17–19]. Recent studies using miR146a-deficient mice have reported that miR146a acts as a brake, attenuating the innate immune response by inhibiting signaling pathways, including Toll-like receptor (TLR), and RIG-I-like receptor (RLR) pathways [20–22]. In human monocytes, miR146a is induced by LPS stimulation in a NF- κ B-dependent manner and inhibits the innate immune responses by targeting TNF receptor-associated factor 6 (TRAF6) and IL-1 receptor associated kinase 1 (IRAK1) genes [16]. In chronically HCV-infected individuals, miR146a has been shown to be up-regulated as a feedback mechanism to prevent an over-stimulated inflammatory state [23–27]. However, the precise mechanism

for miR146a induction in monocytes and its role in regulation of T cell differentiation during HCV infection remain to be elucidated.

In this study, we demonstrated an increase of miR146a expression in monocytes from chronically HCV-infected individuals, and in monocytes incubated with HCV-infected hepatocytes, with or without TLR stimulation. We also investigated the role of HCV-induced miR146a expression in monocytes in regulating T cell differentiation and the potential mechanism involved in regulating inflammatory cytokines leading to viral persistence.

Materials and Methods

Subjects

The study protocol was approved by the institutional review board of East Tennessee State University and James H. Quillen VA Medical Center (ETSU/VA IRB, Johnson City, TN), which has contributed to a database for the storage of blood samples from HCV-infected individuals for the purpose of viral immunology studies. The study subjects comprised three populations: 1) 42 chronically HCV-infected patients, HCV genotype (70% type 1, 30% type 2 or 3) and viral load (ranging from 12,300 ~ 50,000,000 IU/ml) were performed by Lexington VAMC, and all subjects were virologically and serologically positive for HCV prior to the antiviral treatment; 2) 5 HCV subjects who achieved SVR following antiviral therapy with pegylated interferon plus ribavirin and boceprevir; and 3) 22 healthy subjects (HS, blood buffy coat derived from Key Biologics LLC, Memphis, TN) who were negative for HBV, HCV, and HIV infection. Written informed consent was obtained from all participants. The majority of the study subjects were male. The mean age of the three populations was comparable ($P>0.05$).

Cell isolation and culture

Human peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Percoll gradients (GE Healthcare, Piscataway, NJ). CD14⁺ monocytes were further purified from PBMCs using anti-CD14 magnetic beads according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA). The cells were cultured in RPMI 1640 medium containing 10% FBS (Atlanta Biologicals, Flowery Branch, GA), 100 IU/ml penicillin and 100 µg/ml streptomycin and 2 mM L-glutamine (Thermo Scientific, Logan, Utah) at 37°C and 5% CO₂ atmosphere. In some experiments, cells were incubated with or without 1 µg/ml LPS and 2.5 µg/ml R848 (Santa Cruz Biotechnology, Santa Cruz, CA) for 6h. RNA was then isolated and used for PCR analysis.

Co-culture of healthy CD14⁺ monocytes with HCV^{+/-} Huh-7 hepatocyte

Transfection of Huh-7 hepatocytes (kindly provided by Dr. T.J.Liang, Liver Section, NIH/NIDDK) with HCV JFH-1 strain (kindly provided by Dr. T. Wakita) was carried out as described previously [28–29]. For the co-culture experiments, HCV^{+/-} Huh-7 hepatocytes were serum-starved for 18h and then activated with rhIFN-γ (0.1µg/ml, R&D Systems) for 48 h to boost the HCV replication [28–29]. Activated hepatocytes were recovered by 0.05% trypsin-EDTA, and then plated at 5×10^5 cells/well in a 12-well plate. Purified healthy CD14⁺ monocytes were added to the adherent hepatocytes in RPMI media, incubated for

different times, then stimulated with LPS/R848 for 6h, and RNA was isolated and analyzed by RT-PCR.

Measurement of miRNA levels by Real-time RT-PCR

Total RNAs were isolated from monocytes using RNAzol (Molecular Research Center, Inc, Cincinnati, OH) according to the manufacturer's protocol. miRNA levels were quantified by real-time RT-PCR using specific miRNA assay primer sets and TaqMan Universal Master Mix (both from Applied Biosystems). Specific primers for miR146a, miR155-5p, and U6 small nucleolar RNA (snRU6) were obtained from Applied Biosystems. Real-time RT-PCR was performed using a 4800 PCR machine (Bio-Rad System) and miR146a/miR155 values, normalized to snRU6 levels, are expressed as either relative amounts or fold changes using the 2^{-ct} or $2^{-\Delta ct}$ quantification method.

Monocyte transfections

The CD14⁺ monocytes from HCV patients and HS were transfected with 30 pmol of miR146a inhibitor or the anti-miR negative control (Life technologies, Grand Island, NY) using the Human Monocyte Nucleofector Kit and Nucleofector I Device (Lonza, Allendale, NJ). Transfection efficiency was approximately 60% for primary monocytes (50% in naïve CD4⁺ T cells and 20% in nature killer cells) as determined by the transfection of a fluorescently-labeled negative miRNA control. After transfection, monocytes were cultured in IMEM medium (Lonza, Allendale, NJ) supplemented with 10% FBS, 100 ng/ml IL-4 (Peprotech, Rocky Hill, NJ) and 75 ng/ml GM-CSF (Peprotech) for 24–48 h. The monocytes were stimulated with LPS/R848 for 6 h prior to harvest. The supernatants and cell pellets were harvested for cytokine measurement and Western blot analysis, respectively.

Cytokine measurements

IL-23, IL-10, TGF- β 1, IL-12, IFN- α and IFN- β productions were measured in the culture supernatants of HCV patient as well as HS monocytes that were treated with miR146a inhibitor or negative control, using commercially available ELISA kits (R&D, Minneapolis, MN) according to manufacturer's instructions.

Western blot analysis

The transfected monocytes from HCV-infected individuals and HS were lysed on ice in RIPA lysis buffer (Boston BioProducts Inc, Ashland, MA) in the presence of protease inhibitors (Thermo Scientific, Rockford, IL). Cell lysates were centrifuged for 10 min at 4°C, supernatants were recovered and the protein concentrations were measured by Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL). Proteins were separated by SDS-PAGE, and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% milk, 0.5% Tween-20 in Tris buffered saline (TBS), and incubated with the appropriate primary antibodies according to the manufacturer's instruction (anti-SOCS1: Millipore, Temecula, CA; anti-phosphorylated STAT3 and anti-phosphorylated STAT1: Cell Signaling Technology, Inc, Danvers, MA). Appropriate horseradish peroxidase-conjugated secondary antibody (Cell Signaling) was then used and proteins were detected using enhanced chemiluminescence assay kit (Amersham, Piscataway, NJ). Membranes were

stripped and re-probed with anti- β -actin antibody as an internal control (Sigma-Aldrich, St. Louis, MO). Protein bands were captured and quantitatively analyzed by Chemi Doc™ MP Imaging System (Bio-Rad System).

Co-culture of CD14⁺ monocytes interfering miR146a with CD14⁻ PBMCs from HCV-infected individuals

CD14⁺ monocytes isolated from HCV-infected individuals and HS were transfected with miR146a inhibitor or anti-miR negative control and then cultured in IMEM medium (Lonza, Allendale, NJ) supplemented with 10% FBS, 100 ng/ml IL-4 (PeproTech, Rocky Hill, NJ) and 75 ng/ml GM-CSF (PeproTech), as described above. After 24 h, the transfected monocytes were co-cultured with autologous CD14⁻ PBMCs in the presence of 50 ng/ml IL-2 (eBioscience, San Diego, CA) for 5 days, followed by stimulation with 100 ng/ml of PMA (InvivoGen, San Diego, CA) and 1 μ g/ml ionomycin (Invitrogen) for 6 h, with brefeldin A (Biolegend, San Diego, CA), which was added 5 h prior to cell harvest to inhibit cytokine secretion. Cells were analyzed by flow cytometry for Th17 and T-reg cells.

Flow cytometry

Specific antibody direct conjugates was carried out using CD4-PE, CD4-APC and CD25-Alexa488 for cell surface staining, followed by IL-17A-PE for intracellular staining or Foxp3-Pecy5 for transcription factor staining. All antibodies were purchased from eBioscience. IL-17A and Foxp3 staining were carried out using Inside Stain kit and FoxP3 Staining Buffer Set (Miltenyi Biotec), respectively, according to the manufacturer's instructions. The fluorescence minus one (FMO) strategy and isotype controls were used to adjust multicolor compensation for cell gating and determine background levels. The cells were collected on an Accuri C6™ flow cytometer (BD, Franklin Lakes, NJ) and analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

Statistical analysis

The data were expressed as mean \pm SE or median with interquartile, depending on the characteristics of the data distribution. Comparisons between groups were made using one-way analysis of variance (ANOVA). Independent t test or paired t test was used to compare the difference of mean between each two groups. Mann-Whitney test was used to compare the difference of median between each two groups. *P < 0.05 or **P < 0.01 were considered statistically significant or very significant.

Results

miR146a is up-regulated in monocytes from chronically HCV-infected individuals

Previous studies reported the up-regulation of miR146a in different immune cells from HCV-infected individuals [23–27]. To analyze the induction of miRNA146a in monocytes during HCV infection, we measured miR146a levels in CD14⁺ monocytes isolated from PBMCs of 12 chronically HCV-infected patients, 5 SVR individuals, and 4 HS, using real-time PCR. As shown in Fig. 1A (left panel), miR146a expression in monocytes from HCV-infected patients was up-regulated more than 3- to 6-fold compared to SVR and HS, respectively. Notably, miR146a levels were decreased in SVR subjects following antiviral

therapy, but were not completely restored to the levels seen in HS. Since miR146a expression is induced via TLR stimulation [16], we also observed its induction in monocytes stimulated with LPS and R848. Following TLR stimulation, miR146a levels were 2-fold higher in HCV patients than in HS, whereas no significant differences in its levels were observed between SVR and HS (Fig. 1A, right panel).

In order to illustrate the effect of LPS/R848 stimulation on miR146a expression in monocytes, we dynamically measured miR146a levels at various time points following TLR stimulation. Notably, miR146a expression in monocytes from HCV patients was significantly higher than HS with TLR stimulation (Fig. 1B). We also observed the trend of miR146a expression in monocytes with and without LPS/R848 stimulation in HCV patients, SVR, and HS, respectively. Interestingly, while the miR146a expression in monocytes from individuals without HCV infection increased upon LPS/R848 stimulation (Fig. 1C and 1D), which is consistent with the notion of miR146a being a LPS-responsive gene [16], its expression was significantly decreased in monocytes from chronically HCV-infected individuals following 6 h TLR stimulation (Fig. 1E), although these changes were not statistically significant. These findings suggest that miR146a is up-regulated in monocytes from patients with HCV infection and that their response to TLR stimulation is different from monocytes from individuals without HCV infection.

miR146a is up-regulated in monocytes co-cultured with hepatocytes expressing HCV

The increases in miR146a expression in monocytes during HCV infection might be secondary to inflammation stimulation rather than directly due to HCV exposure. To further demonstrate whether HCV plays a primary role in inducing miR146a expression, we performed co-culture experiments by incubating monocytes from 3 HS with HCV⁺ Huh7 cells or HCV⁻ Huh7 cells for various time points (6 h, 12 h, 24 h and 48 h) with or without LPS/R848 stimulation, followed by measuring miR146a expression. As shown in Fig. 2A and 2B, we found that miR146a expression in CD14⁺ monocytes co-cultured with HCV⁺ Huh7 cells was higher at all time points than in monocytes co-cultured with HCV⁻ Huh7 cells, regardless of TLR stimulation. Of note, significant differences were observed only at 6 h and 24 h.

In addition, by comparing miR146a expressions in monocytes with and without TLR stimulation, we found that miR146a expression was increased in monocytes co-cultured with HCV⁻ Huh7 cells with LPS/R848 stimulation compared to non-stimulated monocytes (Fig. 2C). However, miR146a expression was decreased in monocytes co-cultured with HCV⁺ Huh7 cells following 6h LPS/R848 stimulation (Fig. 2D), although these changes were not statistically significant. These data are consistent with the results observed in the *ex vivo* studies by isolated cells from HCV patients and HS (Fig. 1), suggesting that HCV infection alters miR146a expression in monocytes in response to TLR stimulation.

miR146a blockade in CD14⁺ monocytes from HCV-infected individuals inhibits IL-23, IL-10 and TGF- β 1 production

Human monocytes are able to produce inflammatory cytokines (IL-12, IL-23, IL-10 and TGF- β 1) in response to pathogenic infection [30], and miRNAs can regulate these

cytokines, thus directing the differentiation of T lymphocytes [31–32]. In HS, it has been reported that early activation of developing monocyte-derived dendritic cells (MoDCs) allowed only a transient pro-inflammatory cytokine production that was followed by the down-regulation of IL-12 secretion through miR146a/SOCS1/STAT3 feedback regulations [33]. In HCV infection, we have shown an aberrant secretion of IL-23 and IL-10 by monocytes, which contribute to the differentiation of Th17 cells and accumulation of Foxp3⁺ regulatory T cells (T-regs) by SOCS1/STAT3 signaling pathways [7–10]. To better understand the role of miR146a in human monocyte functions during chronic viral infection, we blocked miR146a using an anti-miR146a specific inhibitor and then measured IL-23, IL-10, TGF- β 1, IL-12, IFN- α and IFN- β protein levels in the culture supernatants of treated CD14⁺ monocytes from HCV-infected patients and HS using ELISA. As shown in Fig.3, miR146a inhibition followed by LPS/R848 stimulation in CD14⁺ monocytes from HCV patients resulted in significant decreases in IL-23, IL-10, and TGF- β 1 levels compared with cells transfected with negative control. However, these changes were not observed in CD14⁺ monocytes from HS with the same treatment. In addition, the levels of type I IFN- α and IFN- β were unmeasurable under these conditions (data not shown). These results suggest that miR146a positively regulates IL-23, IL-10 and TGF- β expressions by monocytes during HCV infection.

miR146a regulates monocyte function during HCV infection through inhibition of SOCS1 and induction of STAT3

We have previously demonstrated that HCV core-induced differential regulation of T and B lymphocyte responses and inhibition of IL-12 expression in monocytes is mediated by regulation of JAK/STAT signaling through induction of SOCS1, which is a negative modulator of the JAK/STAT pathway [28–29, 34–36]. It is also reported that IL-12 production by MoDCs from HS is feedback regulated by miR146a/SOCS1/STAT3 pathways [33]. To further assess whether HCV-induced miR146a controls IL-23, IL-10 and TGF- β 1 production in monocytes through regulating SOCS1/STAT pathways, we analyzed SOCS1, STAT1 and STAT3 expressions by Western blot following blockade of miR146a expression in monocytes isolated from HCV-infected individuals and HS. As shown in Fig. 4A, the expression of SOCS1 in monocytes from HCV patients, but not HS, was significantly increased after inhibiting miR146a, at both 24 h and 48 h post-transfection, when compared to those treated with the negative control (Fig. 4A and 4B). Meanwhile, STAT3 phosphorylation in monocytes from HCV patients, but not HS, was significantly decreased by miR146a inhibition at 48 h post transfection (Fig. 4A and 4C), whereas STAT1 phosphorylation was not significantly changed in treated monocytes from both HCV and HS (Fig. 4A and 4D). These data suggest that miR146a may counter-regulate SOCS1 expression and subsequently cease its inhibitory effect on STAT3, thus increasing IL-23, IL-10 and TGF- β 1 productions during HCV infection.

miR146a up-regulation in monocytes expands regulatory T cells during HCV infection

We have previously shown that HCV can differentially regulate IL-12/IL-23/IL-10 expressions in innate immune cells, a milieu prone to the CD4 T differentiation of TH17 cells and Foxp3⁺ T-regs in acquired immune responses [7–10]. To determine whether the increases in IL-23/IL-10/TGF- β production in monocytes by miR146a drives the

differentiation of TH17 cells and generation of T-reg cells during HCV infection, CD14⁺ monocytes isolated from 14 chronically HCV-infected individuals and 6 HS were transfected with miR146a inhibitor or negative control, and then co-cultured with autologous CD14⁻ PBMCs *ex vivo* for 5 days, followed by measuring CD4⁺ IL-17⁺ TH17 cells and CD4⁺CD25⁺Foxp3⁺ T-regs by flow cytometry. We did not observe any significant changes in TH17 cell frequencies after miR146a inhibition in monocytes co-cultured with T cell enriched compartment (data not shown). However, we observed a significant decrease in T-reg cell frequencies in T cells co-cultured with HCV patients' monocytes that were transfected with miR146a inhibitor, but not HS monocytes with the same treatment (Fig. 5A). Taken together, these results suggest that HCV-mediated miR146a induction in monocytes promotes regulatory cytokine expression and T-reg cell development, likely through SOCS1 and STAT3 signaling pathway, which have been shown to be dysregulated during chronic viral infection and contribute to immune suppression and viral persistence [28–29, 34–36].

Discussion

Innate immunity is the first line of host defense, evolved to recognize pathogen-associated molecular patterns (PAMPs) by microorganisms [37]. PAMPs are recognized via cellular receptors (such as TLR, RLR) expressed in immune cells and induce a wide spectrum of cytokine gene expressions, which in turn initiate and shape the inflammatory and adaptive immune responses. Following TLR activation, the adaptor protein MyD88 and IRAK1/TRAF6 are recruited into a signaling molecule complex, which activates the downstream NF- κ B transcription factor and results in up-regulation of immune response genes [38], including type I IFN (IFN- α , IFN- β) and pro- and anti-inflammatory cytokines (IL-12, IL-23, IL-10, TGF- β) that play pivotal roles in eliminating the invading pathogens. On the other hand, TLRs are double-edged swords as aberrant activation of their signaling can be harmful, causing pathological manifestations of inflammatory or autoimmune disorders. Thus, host innate and adaptive immune responses must be tightly regulated by elaborate mechanisms to control their onset and termination. Alternatively, pathogens that develop persistent infection, such as HCV and HIV, have developed multi-layer strategies to evade or subvert the immune responses for their survival or persistence within the host cells. The mechanisms for this immune evasion have yet to be fully understood.

miRNAs are an important class of small (18–25 nt), non-coding RNAs that can regulate gene expression through translational repression or target mRNA degradation [13–15]. More than 700 miRNAs have been identified in humans; some of them are widely expressed while others exhibit only limited developmental stage-, tissue- or cell type- specific expression patterns, and many of these miRNAs are involved in diverse biological processes, such as cytokine expression and cell differentiation [39–41]. As an ever-evolving strategy, viruses may be able not only to modulate cellular miRNA levels but also to interfere with the overall miRNA biogenesis. In particular, miR146a has been shown to be up-regulated in the serum, PBMCs, splenic marginal zone lymphoma (SMZL), and intra-graft of liver transplantation in HCV-infected individuals [23–27]. Despite these observations, little is known regarding how miR146a is induced in monocytes and what role it may play in regulation of T cell differentiation during HCV infection.

Our study demonstrates that miR146a up-regulation by HCV infection plays a pivotal role in regulating inflammatory cytokine production in monocytes, likely via targeting SOCS1 and STAT3 signaling molecules and, subsequently, affecting T-reg differentiation during chronic viral infection. It has been reported that NF- κ B-dependent miR146a induction inhibits innate immune responses by targeting the signaling proteins IRAK1, IRAK2, and TRAF6 [16, 42]. This may be a feedback mechanism whereby microbial components induce NF- κ B activation through a MyD88-dependent pathway, resulting in the up-regulation of miR146a expression, which in turn reduces NF- κ B activity by down-regulating IRAK1/2 and TRAF6 proteins. It is noteworthy that in this scenario, the TRIF-dependent, anti-viral IFN pathway induced by TLR4 remains intact [16]. In our experimental system, the expression levels of type I IFN- α and IFN- β in monocytes isolated from chronically HCV-infected individuals remained undetectable after stimulation with LPS/R848 *ex vivo*, likely reflecting the immunosuppressed state of chronic HCV infection. However, the expression of the inflammatory cytokines IL-23, IL-10, and TGF- β and the frequencies of T-regs that have been shown to be elevated during chronic HCV infection [7–10] were significantly reduced after miR146a inhibition.

By means of computational miRNA target prediction algorithms, potential targets of miR146a other than IRAK1/2, IRF5, and TRAF6 have been identified [16]. Thus, miR146a, like many other miRNAs, may target a wide spectrum of genes that could be involved in regulation of multiple independent cell signaling processes, such as STAT family. We have previously shown that HCV inhibits immune responses by regulating SOCS1 and STAT expression [28–29, 34–36]. Here, we further demonstrated that HCV appears to regulate SOCS1 and STAT3 expression through a miR146a-mediated signaling mechanism. Notably, miR155 has been shown to be upregulated in HCV-infected cells, and miR155 is also known to regulate SOCS1 expression [43–50]. One question is whether miR146a may affect SOCS1 expression through regulating miR155 expression. To answer this question, we examined the miR155 levels in monocytes transfected with miR146a antagonists or negative controls, and the data showed that miR155 was not affected by miR146a transfection (data not shown), suggesting that miR146a may alter the SOCS1 expression through other mechanisms, rather than via miR155. While our study does not identify SOCS1 or STAT3 as the direct target of miR146a, the present data suggest a role for miR146a in controlling inflammatory cytokine production by regulating SOCS1 and STAT3 expression in monocytes, likely through an indirect mechanism, thus directing T cell differentiation and balancing the immune clearance and immune injury during chronic viral infection.

Foxp3⁺ T-reg cells maintain immune homeostasis by limiting different types of inflammatory responses. It has been reported that miR146a is prevalently expressed in T-reg cells and critical for their suppressor function [19]. Previous studies showed that the deficiency of miR146a in T-reg cells resulted in a breakdown of immunological tolerance manifest as a fatal IFN γ -dependent, immune-mediated pathologic lesion in a variety of organs, likely due to augmented phosphorylation of STAT1 [19]. Likewise, heightened STAT1 activation following selective ablation of SOCS1 was associated with similar T-reg-mediated control of Th1 responses and autoimmune pathology [19]. Our results indicate that the differentiation of suppressor T-reg cells is controlled by the same single miRNA, miR146a, which is expressed in monocytes and induced by chronic viral infection through

the SOCS1/STAT3-mediated regulatory cytokines (IL-23/IL-10/TGF- β). Thus, our findings suggest that over-expression of miR146a in innate immunity cells can ensure T-reg cell development by maintaining an optimal threshold of cytokine receptor-dependent activation of transcription factors crucial for a particular type of immune response; in the case of chronic HCV infection, there is a Th2-type response, immune tolerance, and thus viral persistence. Based on our findings for miR146a in chronic HCV infection and other reports in HS, we propose a model (Fig. 5B) for HCV-induced, NF- κ B-mediated miR146a induction in regulating cytokine production and T-regs development. HCV infection induces NF- κ B activation and miR146a expression, which drives inflammatory cytokine (IL-23, IL-10, TGF- β) production by monocytes and subsequently prompts Foxp3⁺ T-reg differentiation. miR146a may also regulate TLR and cytokine signaling through a negative feedback loop involving SOCS1 and STAT3 pathway during chronic viral infection.

Notably, miR146a expression and regulation in monocytes from HCV-infected and uninfected individuals respond differently to TLR stimulation, in that miR146a levels were decreased and positively regulated cytokine productions by monocytes in HCV-infected patients; whereas miR146a levels increased and played a negative role in uninfected individuals [33] following TLR stimulation. This may have resulted from a paradoxical feedback regulatory effect of HCV and TLR on miR146a expression. While HCV or LPS alone can induce miR146a expression, they also trigger the expression of RNA exoribonuclease (such as ERI1) that binds to the 3' end of histone mRNAs and degrades them. We have previously shown HCV delivers negative signaling to NF- κ B as well as AP-1 pathways, and we have recently found that ERI1 is significantly up-regulated in monocytes from HCV-infected individuals following TLR stimulation (data not shown). Therefore, over-activation of monocytes by both HCV and TLR stimulations may lead to triggering of ERI1 feedback mechanisms for immune homeostasis, perhaps explaining the differential expression and regulatory effect of miR146a in monocytes we observed in HCV-infected versus uninfected individuals in response to TLR stimulation. Another possibility is that the different levels of miR146a and its response to TLR stimulation might be related to the monocyte subset (classical, intermediate, non-classical monocytes, or M1 vs M2 monocyte) alterations in HCV-infected individual vs HS, which is an interesting question that need to be addressed in future studies.

In this study, we suggest that excessive activation of STAT3 is kept in check by SOCS1, which is double-checked by miR146a to ensure appropriate immune responses *in vivo*. It may be that HCV-mediated up-regulation of miR146a fine-tunes the TLR and cytokine signaling pathways rather than totally blocking these signals. Nevertheless, the counter-regulatory effects of miR146a on TLR and cytokine signaling may balance immune signaling, fine-tuning immune-mediated viral clearance and host injury. Therefore, we conclude that miR146a may regulate SOCS1/STAT3 signaling and cytokine expression in monocytes, thus directing T cell differentiation and balancing immune clearance and immune injury during chronic viral infection.

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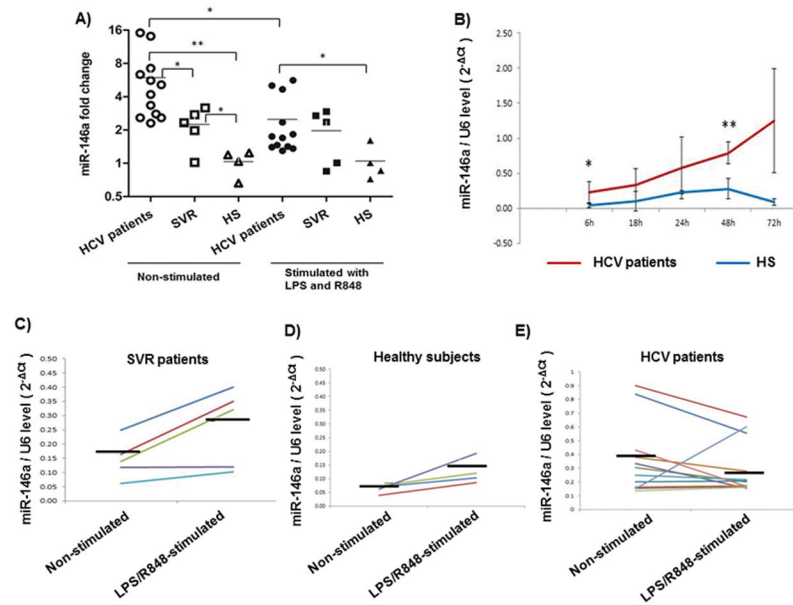


Fig. 1. miR146a is up-regulated in monocytes from chronically HCV-infected individuals CD14⁺ Monocytes were purified from PBMCs from 12 HCV-infected subjects, 5 HCV SVR subjects following anti-viral treatment and 4 HS, stimulated with or without the TLR ligands LPS and R848 for 6 h, followed by real-time RT-PCR analysis of miR146a levels. (A) miR146a fold-changes (2^{-C_t}) from HCV patients and SVR individuals relative to HS, without and with TLR stimulation, are shown. Each symbol represents one individual. Horizontal bar represents the median value. * $P < 0.05$; ** $P < 0.01$, analyzed by Mann-Whitney test. (B) miR146a expression in monocytes from HCV patients versus HS following stimulation by LPS and R848 for indicated times. Error bars are shown from repeated experiments from multiple subjects. * $P < 0.05$; ** $P < 0.01$, analyzed by independent t test. (C–E) Relative miR146a expression level, normalized by U6 (2^{-C_t}), in monocytes from SVR, HS, and HCV subjects before and after TLR stimulation. The horizontal bars represent median values. No significant difference between these changes, analyzed by Mann-Whitney test.

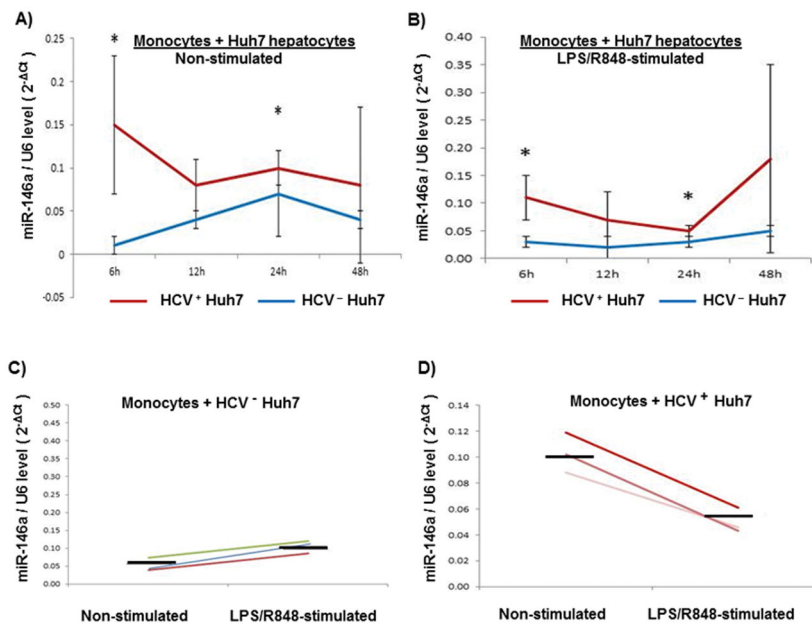


Fig. 2. miR146a is up-regulated in monocytes co-cultured with HCV^{+/-} hepatocytes
 Monocytes were isolated from HS (n=3) and co-cultured with HCV⁺ or HCV⁻ hepatocytes, followed by real-time RT-PCR analysis of miR146a expression. (A–B) Relative changes in miR146a levels, normalized to U6 (2^{-Ct}), in monocytes co-cultured with HCV⁺ hepatocytes versus HCV⁻ hepatocytes with or without LPS and R848 stimulations for the indicated times. *P<0.05, analyzed by independent t test. (C–D) Relative changes in miR146a level, normalized to U6 (2^{-Ct}), in monocytes co-cultured with HCV⁺ Huh 7 or HCV⁻ Huh 7 hepatocytes, respectively, after stimulation with LPS and R848. The horizontal bars represent median values. No significant difference between these changes, analyzed by Mann-Whitney test.

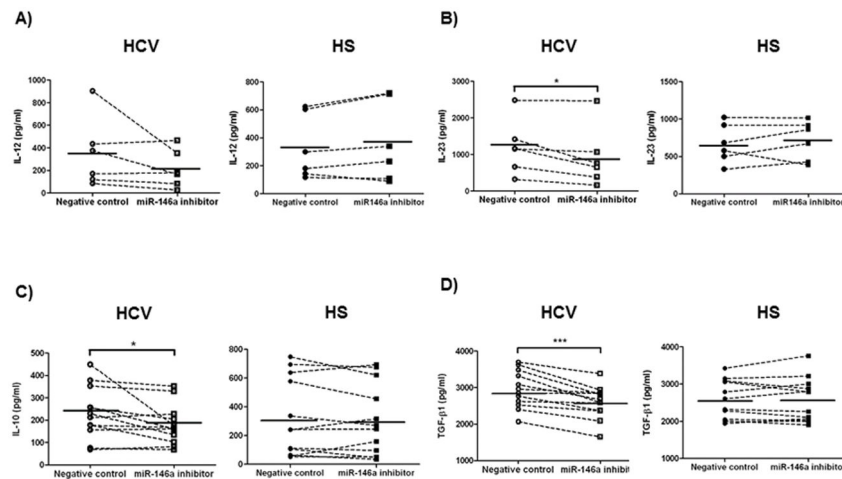


Fig. 3. Effect of miR146a inhibition on inflammatory cytokine production by monocytes from chronically HCV-infected individuals and HS

CD14⁺ monocytes isolated from chronically HCV-infected individuals and HS were transfected with miR146a specific inhibitor or negative control for 24h, and then stimulated with LPS/R848 for 6h prior to harvest. Levels of IL-12 (A), IL-23 (B), IL -10 (C), and TGF- β (D) cytokine production in the culture supernatants was measured by ELISA. Each symbol represents one subject. Data from the same subject, where cells were treated with negative control or miR146a inhibitor, are connected by a dashed-line. Horizontal bars represent the mean value. *P<0.05; ***P<0.001, analyzed by paired t test.

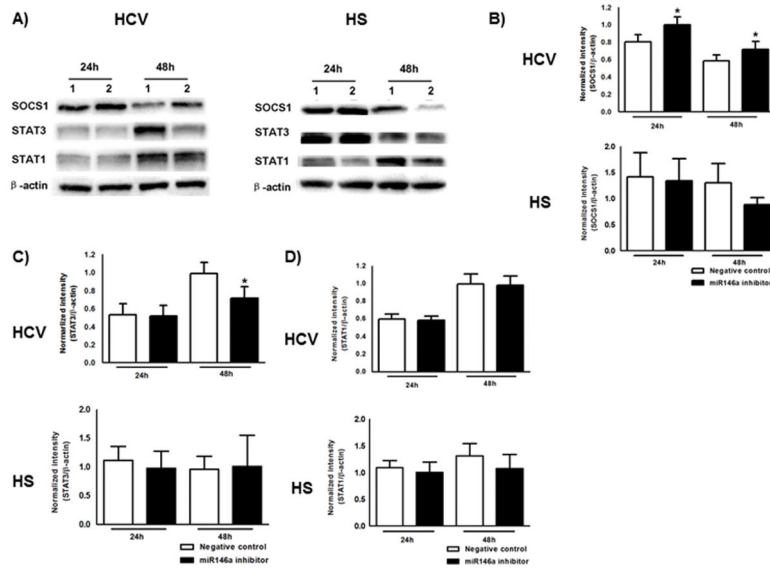


Fig. 4. Effect of miR146a inhibition on the expression of SOCS1, STAT3 and STAT1 in monocytes from chronically HCV-infected individuals and HS

(A) Representative Western blots showing the protein levels of SOCS1, STAT3, and STAT1 in CD14⁺ monocytes from HCV-infected subjects as well as HS transfected with control (1) or miR146a specific inhibitor (2) for 24 h and 48 h and then stimulated with LPS/R848 for 6 h prior to harvest. Membranes were stripped and re-probed for β-actin as a loading control. (B–D) Densitometric analysis of the band intensities shown in A after normalization to β-actin levels. Images were captured and analyzed by Chemi Doc™ MP Imaging System. Data are presented as mean ± SD. *P<0.05, analyzed by independent t test.

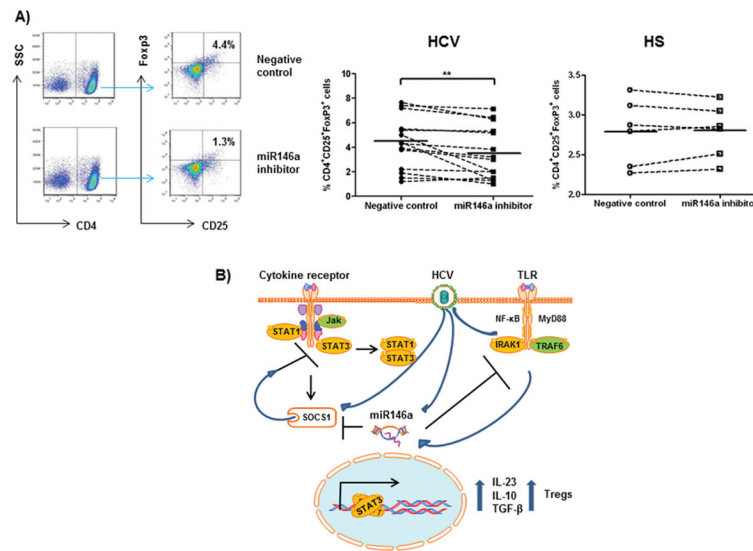


Fig. 5. Effect of miR146a inhibition in monocytes from HCV-infected individuals on Foxp3⁺ T-reg cell development

(A) CD14⁺ monocytes isolated from chronically HCV-infected individuals and HS were transfected with miR146a inhibitor or negative control then co-cultured with autologous CD14⁻ PBMCs for 5 days, followed by flow cytometric analysis of CD4⁺CD25⁺Foxp3⁺ T-reg frequencies. Representative dot plots for the gating of CD4⁺ cells, and then CD25⁺Foxp3⁺ T cells, in the experimental and control group are shown above; and summary data from 14 HCV-infected patients and 6 HS are shown. Each symbol represents one particular subject. Data from the same subject, where cells were treated with negative control and miR146a inhibitor, are connected by a dashed-line. Horizontal bar represents the mean value. **P<0.01, analyzed by paired t test. (B) Proposed model for HCV-induced, NF-κB-mediated miR146a induction and its role in regulating cytokine production and T-reg development during viral infection. HCV infection induces NF-κB activation and miR146a expression in monocytes, which in turn induces inflammatory cytokine (e.g., IL-23, IL-10, TGF-β) and thus promotes Foxp3⁺ T-reg differentiation. miR146a may regulate TLR and cytokine signaling through a negative feedback loop involving SOCS1 and STAT3 signaling pathway during chronic viral infection.