IL-10 plays a central regulatory role in the cytokines induced by hepatitis C virus core protein and polyinosinic acid:polycytodylic acid

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ABSTRACT

Hepatitis C virus (HCV) can cause persistent infection and chronic liver disease, and viral factors are involved in HCV persistence. HCV core protein, a highly conserved viral protein, not only elicits an immunoresponse, but it also regulates it. In addition, HCV core protein interacts with toll-like receptors (TLRs) on monocytes, inducing them to produce cytokines. Polyinosinic acid:polycytodylic acid (polyI:C) is a synthetic analogue of double-stranded RNA that binds to TLR3 and can induce secretion of type I IFN from monocytes. Cytokine response against HCV is likely to affect the natural course of infection as well as HCV persistence. However, possible effects of cytokines induced by HCV core protein and polyI:C remain to be investigated. In this study, we isolated $CD14^+$ monocytes from healthy donors, cultured them in the presence of HCV core protein and/or polyI:C, and characterized the induced cytokines, phenotypes and mechanisms. We demonstrated that HCV core protein- and polyI:C-stimulated $CD14^+$ monocytes secreted tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-10, and type I interferon (IFN). Importantly, TNF-α and IL-1β regulated the secretion of IL-10, which then influenced the expression of signal transducer and activator of transcription 1 (STAT1) and interferon regulatory factor 1 (IRF1) and subsequently the production of type I IFN. Interestingly, type I IFN also regulated the production of IL-10, which in turn inhibited the nuclear factor (NF)-κB subunit, reducing TNF-α and IL-1β levels. Therefore, IL-10 appears to play a central role in regulating the production of cytokines induced by HCV core protein and polyI:C.

1. Introduction

Hepatitis C virus (HCV) infection in the majority of affected individuals becomes chronic, and the mechanisms responsible for persistent HCV infection are not completely understood. It has been suggested that the host's insufficient immune response and particularly weak cellular immunity lead to viral persistence [1,2]. However, viral factors are also involved in HCV pathogenesis.

Accumulating evidence suggests that HCV proteins can suppress the host's immune responses by interfering with immune cell functions [3–5]. The core protein is a highly conserved structural protein that is required to assemble the viral nucleocapsid, which encapsulates the viral RNA genome. HCV core protein also interacts with HCV envelope proteins to initiate virus particle assembly [6,7]. In addition, it regulates cellular transcription, transformation, signal transduction, and innate immunity. Moreover, HCV core protein is believed to engage in virus-mediated pathogenesis through modulating apoptosis, cell growth, and reactive oxygen species production [6,8]. HCV core-dependent deregulation of the Janus kinase-signal transducer and activator of transcription (STAT) signaling pathway, which suppresses HCV-induced innate immunity, has been suggested [9–11].

Monocytes are important immune cells that defend the host against numerous pathogens as well as initiate and control adaptive immunity [12]. These cells express a panel of receptors including toll-like receptors (TLRs), which bind to diverse pathogen-derived products, thus triggering the immune response to a broad range of bacterial and viral pathogens [13,14]. HCV core protein and double-stranded RNA can interact with TLR2 and TLR3 on monocytes and induce large quantities of proinflammatory and anti-inflammatory cytokines, which may lead to hepatic inflammation that can facilitate HCV infection clearance.

Peripheral monocytes produce interleukin (IL)-10, which can inhibit the HCV immune response [15,16]. The IL-10 level has been associated with an elevated serum alanine transaminase and/or histological

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grade in HCV patients [17]. In addition, it has been correlated with outcomes of chronic viral infection [18]. One study has revealed that type I interferon (IFN) can induce IL-10 production and the subsequent STAT3 phosphorylation, thus further inhibiting the expression of proinflammatory cytokines including IL-1β, tumor necrosis factor-α (TNF-α), and IL-12 [19]. It is unclear whether proinflammatory cytokines regulate the IL-10 level and functions or further modulate type I IFN expression. In this study, we investigated the responses of monocytes to HCV core protein and polyinosinic acid:polycytodylic acid (polyI:C) which could simulate HCV RNA. And we showed that proinflammatory cytokines induce the upregulation of IL-10 and the subsequent inhibition of type I IFN production, in turn, type I IFN increase the secretion of IL-10 and then inhibits proinflammatory cytokines production. IL-10 appears to play a central role in the regulatory network of inflammatory and antiviral cytokines.

2. Materials and methods

2.1. Reagents

We purchased the experimental reagents from the following sources: HCV Core Antigen [amino acids 2–192] Recombinant and β-galactosidase (Meridian Life Science, Inc., Saco, ME, USA); polyI:C (Invivogen, San Diego, CA, USA); anti-human IL-10 Functional Grade Purified, Anti-Human TNF alpha Functional Grade Purified, Anti-Human IL-1 beta Functional Grade Purified, Anti-Human IFN beta Functional Grade Purified, and Anti-Human IFN-α (eBioscience, San Diego, CA, USA); human IL-10 ELISA ready-SET-Go, human TNF alpha ELISA ready-SET-Go, human IL-1 beta ELISA ready-SET-Go (eBioscience, San Diego, CA, USA); VeriKine TM Human Interferon beta/alpha ELISA Kit (PBL Assay Science, Piscataway, NJ, USA); CD14 microbeads (Miltenyi Biotec, Inc., Auburn, CA, USA); PE/Cy7-conjugated anti-human HLA-DR, PerCP-conjugated anti-human HLA-DR, PE-conjugated anti-human CD38, PE/Cy7-conjugated anti-human PD-L1 (BD Biosciences, Franklin Lakes, NJ, USA); Human Fc Receptor Binding Inhibitor Purified (eBioscience, San Diego, CA, USA); Easypure RNA kit (Transgen Biotech, Beijing, China); Faststart Universal SYBR Green Master (ROX) (Roche, Basel, Switzerland); and TransScript All-in-One First-Strand cDNA Synthesis SuperMix for qPCR (Transgen Biotech, Beijing, China).

2.2. Isolation of monocytes from peripheral blood

Peripheral blood concentrated lymphocytes of Healthy blood donors were provided by the Changchun Blood Center, and informed consent was obtained according to the protocols of the Changchun Blood Center. Peripheral blood mononuclear cells (PBMCs) were isolated and purified by density gradient centrifugation using lymphocyte separation medium.

2.3. Purity and culture of $CD14^+$ monocytes

The $CD14^+$ cells were isolated and purified using CD14 microbeads. The purities of the $CD14^+$ cells were determined by flow cytometry, and the purity was always >90%. A half million purified $CD14^+$ monocytes were cultured in 96-well flat-bottomed plates with complete RPMI 1640 containing 10% fetal calf serum and 100 IU/mL penicillin and streptomycin at 37 °C with 5% CO_2.

2.4. Flow cytometry

Cell staining and analyses were performed as described previously [20,21]. In brief, $CD14^+$ monocytes were cultured for 2 days in the presence of HCV core protein (final concentration of 10 μg/mL) or polyI:C (final concentration of 50 μg/mL) and then resuspended in staining buffer (phosphate-buffered saline containing 0.5% bovine serum albumin) and preincubated with FcR blocking reagent for 15 min at 4 °C. Next, the cells were stained with PE/Cy7-conjugated HLA-DR or PerCP-conjugated anti-human HLA-DR, PE-conjugated anti-human CD38, or PE/Cy7-conjugated anti-human PD-L1 and incubated for 20–40 min. The cells were then washed with staining buffer and resuspended in phosphate-buffered saline containing 1% paraformaldehyde and analyzed on a flow cytometer (BD LSRFortessa cell analyzer, USA). The acquired data were analyzed with FlowJo (Treestar software).

2.5. Analysis of the cytokines

The cytokine concentrations were measured using ELISA kits. One hundred microliters of supernatant was used for each analysis. All samples were assayed in duplicate.

2.6. qPCR

The $CD14^+$ monocytes were cultured and treated with HCV core protein and polyI:C or neutralizing antibodies (the final concentrations of anti-human IL-10 and IL-1β were 10 ng/μL, anti-human TNF-α was used at 12.5 ng/μL, and anti-human IFN-α and IFN-β were used at 5 ng/μL, according to the efficiency of neutralization by ELISA). The cells were then harvested using an Easypure RNA kit and reverse transcribed into complementary DNA (cDNA) per the manufacturer's instructions. The cDNAs were amplified by qPCR in a Real-Time PCR System (Applied Biosystems StepOnePlus™, Life Technologies, USA). Target gene expression was determined using the comparative cycle threshold (ΔΔCT) method and normalized to GAPDH. The primers are shown in Table 1.

2.7. Statistical analysis

Statistical analyses were performed using SPSS 18.0. Differences were evaluated using one-way analysis of variance (ANOVA), followed by Bonferroni's multiple-comparison test. $p < 0.05$ was considered significant and is indicated by an asterisk (*).

3. Results

3.1. HCV core protein- and polyinosinic acid:polycytodylic acid (polyI:C)-induced secretion of cytokines in $CD14^+$ monocytes

Monocytes constitute 10–20% of the total PBMCs. Monocytes are characterized by their phenotypic CD14 expression, commonly called $CD14^+$ monocytes.

In the present study, we first investigated the expression levels of TLRs on PBMCs and found that the PBMCs expressed TLR2, TLR3, and

Table 1
Primers used for qPCR analysis of gene expression.

Gene	Primers	Product size
GAPDH	PF: 5′-CCACCTTTGACGCTGGG-3′ PR: 5′-CATACCAGGAAATGAGCTTGACA-3′	66 bp
IL-10	PF: 5′-GGGAGAACCTGAAGACCCTCA-3′ PR: 5′-TGCTCTTGTTTTCACAGGGAAG-3′	72 bp
TNF-α	PF: 5′-ATCCTGGGGGACCCAATGTA-3′ PR: 5′-AAAAGAAGGCACAGAGGCCA-3′	112 bp
IL-1β	PF: 5′-CAGAAGTACCTGAGCTCGCC-3′ PR: 5′-AGATTCGTAGCTGGATGCCG-3′	153 bp
IFN-β	PF: 5′-TGGCTGGAATGAGACTATTGTTGAG-3′ PR: 5′-CAGGACTGTCTTCAGATGGTTTATCT-3′	76 bp
STAT1	PF: 5′-CGGCTGAATTTCGGCACCT-3′ PR:5′-CAGTAACGATGAGAGGACCCT-3′	81 bp
STAT3	PF: 5′-ATTGCCCGGATTGTGGCCCG-3′ PR: 5′-CTCCGTCACCACGGCTGCTG-3′	117 bp
IRF1	PF: 5′-AAAAGGAGCCAGATCCCAAGA-3′ PR: 5′-CATCCGGTACACTCGCACAG-3′	128 bp
NFκB	PF: 5′-TGGGCTACACCGAAGCAAT-3′ PR: 5′-GGGCCTGAGAGGTGGTCTT-3′	69 bp

TLR4 (data not shown). Then $CD14^+$ monocytes were separated and cultured alone, or with β-galactosidase (β-gal as control) or with HCV core protein and/or polyI:C. Our previous study showed that the peak secretion of IL-10 occurred on the second day of incubation, but the peaks of TNF-α, IL-1β and IFN-β secretion occurred on the first day [22]. Thus, we detected the IL-10 level in the medium collected on the second day and the levels of TNF-α, IL-1β, and IFN-β in the medium collected on the first day after the addition of HCV core protein or polyI:C. Our results showed that HCV core protein could induce the secretion of TNF-α, IL-1β, and IL-10, and polyI:C can induce secretion of type I IFN in $CD14^+$ monocytes. We also found that HCV core protein inhibited polyI:C-induced type-I IFN production. However, polyI:C acted synergistically with HCV core protein to induce secretion of IL-10 in the monocytes but inhibited HCV core protein-induced TNF-α and IL-1β secretion (Fig. 1).

3.2. HCV core protein and polyI:C affect the phenotypes of $CD14^+$ monocytes

To further characterize the phenotypes of monocytes induced by HCV core protein or polyI:C, flow cytometry was used to determine phenotypic expression, including human leukocyte antigen-D related (HLA-DR), cluster of differentiation 38 (CD38), and programmed death-ligand 1 (PD-L1), which reflect the host's immune response [23,24] related to HCV persistence [25–32].

We found that the optimal concentrations of HCV core protein and polyI:C for induction were 10 μg/mL and 50 μg/mL, respectively, and that the number of dead cells became fewer on day 2, resulting in better phenotypic expression. Our results also showed that HCV core protein inhibited HLA-DR expression but upregulated PD-L1 expression. PolyI:C induced the expression of CD38, HLA-DR, and PD-L1 in $CD14^+$ monocytes. Meanwhile, HCV core protein inhibited polyI:C-induced CD38 and HLA-DR expression. However, HCV core protein synergized with polyI:C to upregulate PD-L1 expression in monocytes. Therefore, HCV core protein and polyI:C could modulate the monocyte phenotypes, which may alter the monocyte functions (Fig. 2).

3.3. HCV core protein and polyI:C modulate the phenotypes of $CD14^+$ monocytes through cytokines

We investigate whether cytokines involved in the regulation on phenotypes of $CD14^+$ monocytes.

Our results indicated HCV core protein- and polyI:C-induced cytokine secretion in $CD14^+$ monocytes. Moreover, these cytokines appear to modulate the monocyte phenotype. Neutralizing antibodies were used to demonstrate the direct contribution of specific cytokines to cell surface marker expression. When IL-10 or TNF-α expression was blocked, HLA-DR expression increased; but when type I IFN was blocked, HLA-DR expression decreased. In addition, the expression of CD38 and PD-L1 was decreased when TNF-α, IL-1β, or type I IFN (Fig. 3) was blocked. Therefore, HCV core protein and polyI:C modulated the phenotypes of $CD14^+$ monocytes via cytokines.

3.4. IL-10 is required for the regulation of cytokines induced by HCV core protein and polyI:C

Given that cytokines can modulate cell phenotypes, next we studied the interaction of IL-10 and other cytokines. Our results revealed that blocking of IL-10 resulted in increased levels of TNF-α, IL-1β, and IFN-β. On the other hand the IL-10 level was decreased when TNF-α, IL-1β, or type I IFN was blocked (Fig. 4A). That is, TNF-α, IL-1β, or type I IFN induced the production of IL-10, and in turn, IL-10 inhibited the secretion of TNF-α, IL-1β, and type I IFN. The quantitative detection of cytokine mRNAs was consistent with the detected protein levels (Fig. 4B).

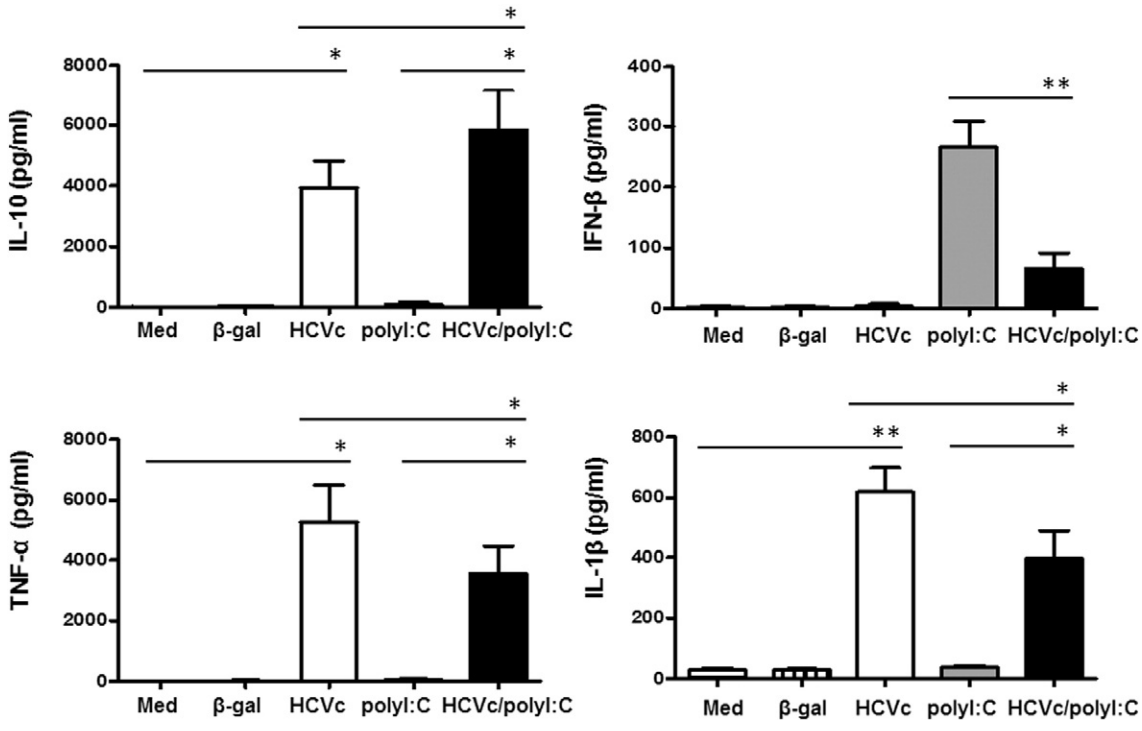

Fig. 1. HCV core protein and polyI:C induce cytokines secretion in $CD14^+$ monocytes. $CD14^+$ monocytes were separated and cultured alone or with β-gal (control) or with HCV core protein and/or polyI:C. The supernatants were collected on the first day for assessment of TNF-α, IL-1β, and IFN-β and on the second day for IL-10, as determined by ELISA. The bar graphs indicate the cytokine levels from four independent experiments. Med, medium; HCVc, HCV core protein; PIC, polyI:C. The error bars represent the standard error of the mean. $^*p < 0.05$, $^{**}p < 0.01$.

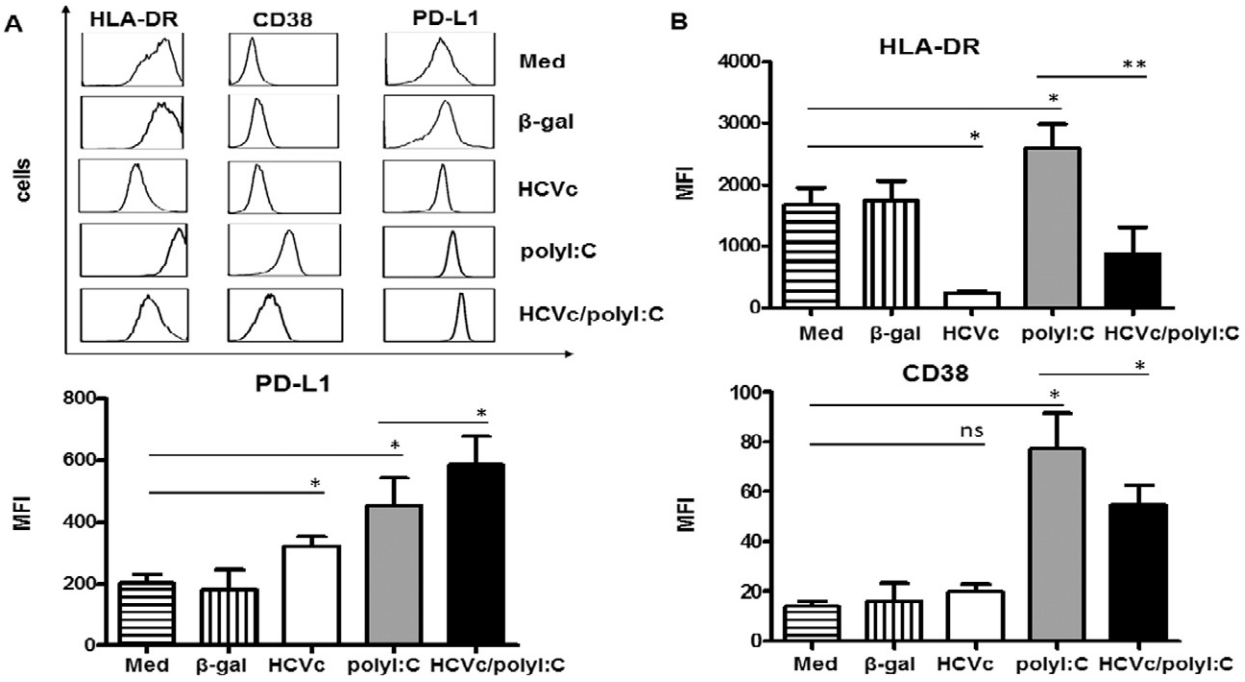

Fig. 2. HCV core protein and polyI:C modulate the phenotypes of $CD14^+$ monocytes. $CD14^+$ monocytes were separated and cultured alone or with β-gal or with HCV core protein and/or polyI:C for 2 days. The cells were then collected and stained with PE/Cy7-conjugated HLA-DR, PE-conjugated anti-human CD38, or PE/Cy7-conjugated anti-human PD-L1 and analyzed by flow cytometry. The histograms (A) and bar graphs indicate the mean fluorescence intensities (MFIs) (B) of four independent experiments. Med, medium; HCVc, HCV core protein; PIC:polyI:C. The error bars represent the standard error of the mean. *$p < 0.05$, **$p < 0.01$, and ns indicates nonsignificant.

3.5. IL-10 regulates the secretion of TNF-α and IL-1β by inhibiting nuclear factor (NF)-κB and the production of type I IFN by inhibiting STAT1 and interferon regulatory factor 1 (IRF1)

We further studied the immunomodulatory mechanism of IL-10. Several candidate transcription factors that are likely involved in IL-10 production by macrophages have been described [33–36]. Our results revealed that the expression levels of NFκB, STAT1 and IRF1 were increased when IL-10 was blocked, as assessed by quantitative polymerase chain reaction (qPCR); however, no significant change was detected in the expression of STAT3 (Fig. 5).

In combination with other studies, our results led us to the conclusion that TNF-α, IL-1β, and type I IFN induced IL-10 secretion and that IL-10 subsequently inhibited type I IFN production by downregulating STAT1 and IRF1, thus inhibiting the secretions of TNF-α and IL-1β by inhibiting NFκB. These factors form a regulatory network centered on IL-10.

4. Discussion

In this study, we examined whether HCV core protein and polyI:C interact with TLRs on monocytes or modulate the biological functions

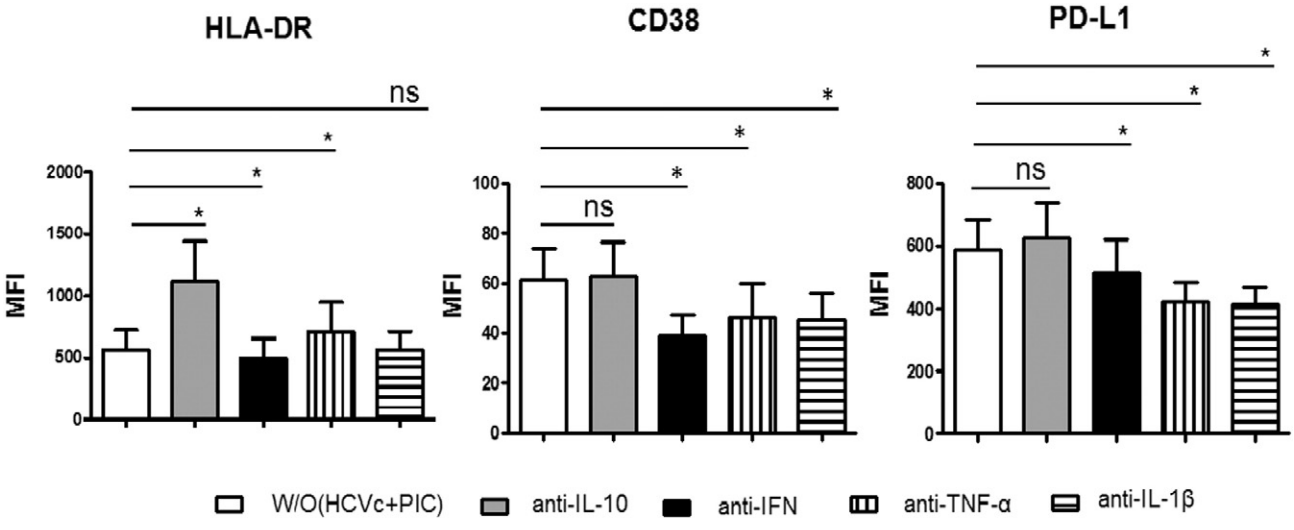

Fig. 3. Cytokines modulate the phenotypes of $CD14^+$ monocytes. $CD14^+$ monocytes were separated and cultured with HCV core protein and polyI:C in the presence or absence of neutralizing antibodies for 2 days. The cells were then collected and stained with PerCP-conjugated HLA-DR, PE-conjugated anti-human CD38, or PE/Cy7-conjugated anti-human PD-L1 and analyzed by flow cytometry. The bar plots of the mean fluorescence intensities (MFIs) of seven independent experiments are shown. W/O stands for "without neutralizing antibody" while all groups received both HCVc and PIC. The error bars represent the standard error of the mean. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ns indicates nonsignificant.

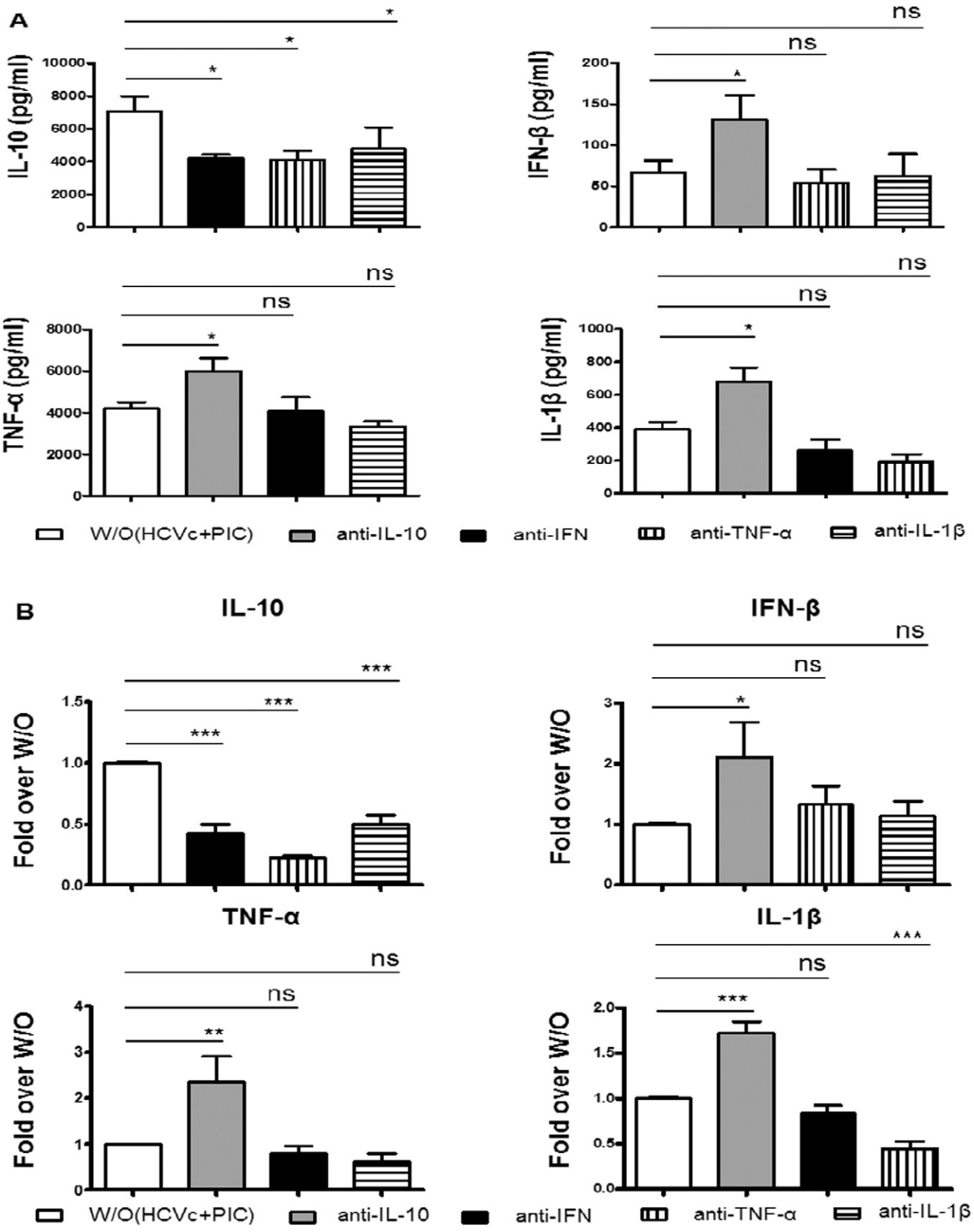

Fig. 4. IL-10 is required for regulating cytokine expression. $CD14^+$ monocytes were separated and cultured with HCVc and polyI:C in the presence or absence of neutralizing antibodies for 1 or 2 days. The supernatants were then collected and analyzed by ELISA. The bar plots indicate the levels of cytokines from four independent experiments (A). The $CD14^+$ monocytes were separated and cultured with HCVc and polyI:C in the presence or absence of neutralizing antibodies for approximately 10 h; the cells were then collected, and the RNA was extracted. The expression of IL-10, TNF-α, IL-1β, and IFN-β mRNAs was assessed by qPCR. The expression levels were normalized with GAPDH, and the fold inductions were calculated relative to the untreated controls (B). W/O stands for "without neutralizing antibody" while all groups received both HCVc and PIC. The error bars represent the standard error of the mean. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and ns indicates nonsignificant.

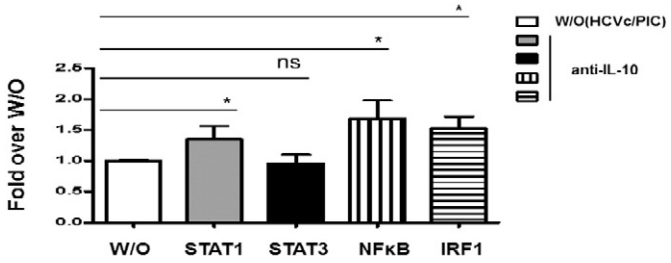

Fig. 5. IL-10 regulates cytokine secretion through negative feedback. $CD14^+$ monocytes were separated and cultured with HCVc and polyI:C in the presence or absence of neutralizing antibodies for 10 h; the cells were then collected, and the RNA was extracted. The expression of STAT1, STAT3, NFκB, and IRF1 mRNA was assessed by RT-PCR. The expression levels were normalized with GAPDH, and the fold inductions were calculated relative to the untreated controls. W/O stands for "without neutralizing antibody" while all groups received both HCVc and PIC. The error bars represent the standard error of the mean. $^*p < 0.05$, and ns indicates nonsignificant.

of monocytes that might directly or indirectly contribute to persistent HCV infection. Many studies showed that HCV core protein bound to TLR2 [37,38], while polyI:C could activate TLR3 [39]. We found that TLR2, TLR3, and TLR4 were expressed on purified $CD14^+$ monocytes (data not shown). Our results also showed that HCV core protein induced the secretion of the inflammatory cytokines TNF-α and IL-1β as well as the immunomodulatory cytokine IL-10. Meanwhile, polyI:C induced type I IFN, which is currently used for treating chronic HCV infection.

Cytokines can regulate both phenotypes and functions of cells. IL-37 induces a phenotypic shift in human acute monocytic leukemia-derived macrophages toward a high-$CD206^+$ and low-$CD86^+$ macrophage subtype [40]. IL-15 strongly induces the expression of CD69 and CD54 in approximately 30% of natural killer cell subsets and downregulates CD62L expression, which is indicative of functional activation [41]. Monocytes that had been treated with IL-10 in vitro had significantly decreased HLA-DR expression, thus resulting in expansion of the $CD14^+$ HLA-$DR^{low/-}$ population [42]. Our results revealed that TNF-α, IL-1β, IL-10 and IFN-βinduced by HCV core protein and polyI:C appeared to modulate the expression of HLA-DR, CD38 and PD-L1, and might alter their biological functions.

Most of important, IL-10 appeared to play a central role in the regulatory circuit of inflammatory and antiviral cytokines. we found that IL-10 not only inhibited the production of TNF-α and IL-1β induced by HCV core protein but also reduced the secretion of type I IFN stimulated by polyI:C. IL-10 may limit hepatic inflammation and injury, but it also potentially slows down HCV infection clearance. Interestingly, we also found that TNF-α, IL-1β and type I IFN elevated IL-10 expression, which in a negative feedback mechanism reduced the production of TNF-α, IL-1β, and type I IFN. Our findings are in agreement with published data that the production and signaling pathway of type I IFN are involved in *lipopolysaccharide*-induced IL-10 production that subsequently suppresses proinflammatory cytokine genes [19].

To gain insight into the mechanisms of IL-10 that regulates these cytokines, several candidate transcription factors have been described. One study reported that type I IFN induced the production of IL-10 and subsequent STAT3 phosphorylation in murine *bone-marrow-derived macrophage*s and additionally inhibited the expression of proinflammatory genes [19]. Another study has found that IL-10 inhibits the expression of both IFN-α- and IFN-γ-induced genes by suppressing tyrosine phosphorylation of STAT1 [43]. We cultured $CD14^+$ monocytes in the presence of HCV core protein and polyI:C. We found that NFκB, STAT1, and IRF1 were a few components that regulate IL-10. Our results suggest that IL-10 might inhibit NFκB, which leads to the suppression of TNF-α and IL-1β while downregulating STAT1 and IRF1, which reduces the type I IFN level. However, the expression of STAT3 was not affected. We believe that this difference was attributable to a species difference.

In conclusion, peripheral monocytes secreted TNF-α, IL-1β, and type I IFN upon induction with HCV core protein and polyI:C. All three cytokines increased IL-10 production, which in a negative feedback mechanism inhibited the expression of proinflammatory cytokines and type I IFN. Our results suggested that IL-10 countered hepatic inflammation and clearance of HCV infection and that it may represent a factor contributing to the persistence of HCV infection.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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