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An immunosuppressive function of interleukin-35 in chronic hepatitis C virus infection

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ABSTRACT

Interleukin (IL)-35, a newly identified member of the IL-12 cytokine family, has been reported to suppress inflammation and induce immunotolerance. However, little is known regarding the role of IL-35 during chronic hepatitis C virus (HCV) infection. Herein, we measured the serum IL-35 concentration of 73 patients with hepatitis C and 22 healthy individuals, as well as further investigated the modulatory function of IL-35 on $CD4^+CD25^+CD127^{dim/-}$ regulatory T cells (Tregs) and on hepatocytes infected with HCV in cell culture (HCVcc). IL-35 expression was significantly increased in patients with chronic hepatitis C and was positively correlated with the levels of HCV RNA. Inhibition of viral replication led to decreases in the serum levels of IL-35. IL-35 stimulation not only elevated the percentage of Tregs but also robustly inhibited cellular proliferation and up-regulated the production of anti-inflammatory cytokines (*e.g.*, IL-10 and IL-35) in a HCV-specific and non-specific manner, which indicates enhancement of the suppressive function of Tregs. Although IL-35 did not exert anti-HCV activity in HCVcc-infected Huh7.5 cells, it reduced inflammatory cytokine secretion from Huh7.5 cells. This was probably *via* inhibition of the STAT1 and STAT3 signaling pathways, which could suppress subsequent liver damage due to chronic hepatitis C. The current data suggested that IL-35 contributes to persistent HCV infection by inhibiting antiviral immune activity. Moreover, IL-35 might also protect against HCV-induced liver injury by down-regulating the expression of proinflammatory cytokines. Thus, the immunosuppressive properties of IL-35 might play contradictory roles in maintaining viral persistence and reducing the inflammatory responses in chronic HCV infection.

1. Introduction

Hepatitis C virus (HCV) is a worldwide public health problem with approximate 170 million persistent infections all over the globe, and can result in end-stage liver diseases, such as liver cirrhosis and hepatocellular carcinoma [1,2]. Although the administration of direct antiviral agents (DAAs) has led to a significant improvement in the sustained virological response (SVR) compared to that elicited by peginterferon plus ribavirin combination therapy [3,4], there are additional issues to contend with, such as viral relapse, immune tolerance and exhaustion, all of which require further study [5]. Moreover, since no DAAs were approved by the Chinese Food and Drug Administration until April 2017, peginterferon plus ribavirin therapy is still the standard treatment for HCV infection in China. Thus, the virological response is varied among patients with chronic hepatitis C patients and different genotypes. Patients with the HCV 1b genotype exhibit a lower virological response rate than those with HCV 2a genotype infection following peginterferon and ribavirin therapy [6].

Although HCV is a non-cytopathic RNA virus, interactions between the virus and the host immune response could induce variable liver damage [7]. Interleukin (IL)-35 is a heterodimeric hematopoietin that belongs to the IL-12 family of cytokines and comprises the IL-12p35 and Epstein-Barr virus-induced gene 3 (EBI3) subunits [8]. IL-35 is highly expressed in $CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs) but not in resting or activated effector $CD4^+$ T cells [9]. Knockdown of IL-12p35 and EBI3 in Tregs decreased the regulatory activity of these cells and failed to control homeostatic proliferation *in vitro* and *in vivo* [9]. Moreover, IL-35 induced Treg proliferation and expansion and suppressed Th17 cell differentiation [10], which can potentially translate to therapeutic applications against autoimmune diseases (*e.g.*, rheumatoid arthritis [11–13], autoimmune diabetes [14], and systemic lupus erythematosus [15]), bacterial infections (*e.g.*, active tuberculosis [16] and sepsis [17]), viral infections (*e.g.*, influenza virus [18,19] and hepatitis B virus (HBV) [20]), and cancers (*e.g.*, hepatocellular carcinoma [21], breast cancer [22], colorectal cancer [23], and pancreatic adenocarcinoma [24]).

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It is well established that Tregs inhibit antiviral immune responses and regulate inflammatory liver injury, both of which contribute to immune tolerance and viral persistence in HCV infection [25]. However, the role of IL-35 in chronic hepatitis C has not been fully elucidated. We hypothesized that IL-35 could modulate the bioactivities of Tregs and hepatocytes, and thus play an immunosuppressive function in chronic HCV infection. To test this possibility, *in vitro* cell cultures of HCV-specific Tregs and HCV-infected hepatocytes were stimulated with recombinant IL-35 to investigate changes in their function.

2. Materials and methods

2.1. Subjects

A total of 73 patients with chronic HCV infection were enrolled in the present study. All the patients were hospitalized or followed-up at the Second Hospital of Jilin University between March 2014 and June 2016. All the enrolled patients were positive for HCV RNA and anti-HCV antibody for at least six months. None of the patients were co-infected with other hepatitis viruses or had end-stage liver diseases (*e.g.*, decompensate cirrhosis, liver failure, and hepatocellular carcinoma). Patients who received antiviral treatment or immunomodulatory therapies before baseline sampling were also excluded from this study. Among the 73 patients with chronic hepatitis C, 25 patients received peginterferon-α2 plus ribavirin. Blood samples were also collected 48 weeks post-treatment and either 12 or 24 weeks following completion of therapy. Patients who could sustain an undetectable viral load at 24 weeks after therapy completion were defined as SVR. For normal controls, twenty-two healthy age- and sex-matched individuals were also enrolled. The clinical characteristics of all the enrolled subjects are shown in Table 1. The study protocol was approved by the ethics committee of the Second Hospital of Jilin University, and conformed to ethical guidelines set by the 1975 Declaration of Helsinki. Written informed consent was obtained from each enrolled subject.

2.2. Virological and biochemical assessments

HCV RNA was quantified using a commercial real-time Polymerase Chain Reaction (PCR)-Fluorescence Quantitative Detection Kit for HCV RNA (DaAn Gene, Guangzhou, Guangdong Province, China) with a detection limitation of 2 log10 copies/mL. HCV genotyping was performed using a second-generation line probe assay (Inno-Lipa II, Innogenetics, Zwijndrecht, Belgium), and serum biochemical assessments were completed using Hitachi 7600 automatic analyzer (Hitachi, Tokyo, Japan).

2.3. Peripheral blood mononuclear cell (PBMC) isolation and Treg purification

PBMCs were isolated using density gradient centrifugation with Ficoll-Hypaque (Sigma-Aldrich, St Louis, MO, USA). $CD4^+CD25^+CD127^{dim/-}$ Tregs were purified using a $CD4^+CD25^+CD127^{dim/-}$ regulatory T cell isolation kit II (Miltenyi, Bergisch Gladbach, Germany) following the manufacturer's instruction. The purity of enriched $CD4^+CD25^+CD127^{dim/-}$ Tregs was > 95% as assessed using flow cytometry.

Table 1
The clinical characteristics of enrolled subjects.

	Chronic hepatitis C	Healthy individuals
Number of enrolled subjects	73	22
Age (years)	31 (18–56)	35 (23–51)
Male/female	43/30	14/8
HCV RNA (log10 copies/mL)	5.87 (3.11–8.89)	Not available
HCV genotype (1b/2a)	47/26	Not available
ALT (IU/L)	57 (11–281)	25 (9–38)

Data were shown as median and range.

2.4. Cell culture

The purified Tregs were treated with recombinant human IL-35 (rIL-35, Peprotech, Rocky Hill, NJ, USA) at a final concentration of 1 ng/mL [26] for 6 h and were co-cultured with autologous $CD4^+CD25^-$ T cells at ratio of 1:1, 1:4, or 1:8 in the presence of either anti-CD3/anti-CD28 (final concentration, 1 μg/mL, eBioscience, San Diego, CA, USA) or a HCV NS3 T helper cell epitope peptide (residues, 1248–1261; sequence, GYKVLVLNPSVAAT; final concentration, 20 μg/mL) [27]. Tregs were washed twice to remove residual IL-35 before co-culturing. Co-cultures were maintained for 72 h, and the medium supernatants as well as the cultured cells were harvested for further analyses.

2.5. Generation of HCV viral stocks and infection of Huh7.5 cells

Infectious HCV in cell culture (HCVcc) was purchased from Zhonghaichao Biotech (Kunming, Yunnan Province, China) and generated as described previously [28]. The HCVcc copy was determined using real-time PCR. Huh7.5 cells were seeded into a 24-well plate, and 10^7 copies of HCVcc were incubated on the cells for 5 h. Infected cells were washed five times with DMEM, and cultured for 48 h in DMEM supplemented with 10% heat-inactivated fetal bovine serum with or without rIL-35 (final concentration, 1 ng/mL).

2.6. Enzyme-linked immunosorbent assay (ELISA)

The concentrations of IL-35 and other related cytokines, such as IL-10, interferon (IFN)-γ, IFN-β, transforming growth factor (TGF)-β, and tumor necrosis factor (TNF)-α were measured using commercial ELISA kits (CUSABIO, Wuhan, Hubei Province, China) following the manufacturer's instructions.

2.7. Cytokine assay

The levels of the following cytokines and chemokines in the cultured supernatants were tested using a Human Mag Luminex Performance Assay Base Kit, HS cytokine A (R & D System, Minneapolis, MN, USA) with a Luminex LX-200 Instrument with xPONENT 3.1 (R & D System) following the manufacturer's instructions: granulocyte-macrophage colony stimulating factor (GM-CSF), IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 p70, TNF-α, and vascular endothelial growth factor (VEGF).

2.8. Flow cytometry

PBMCs were stimulated with rIL-35 (final concentration, 1 ng/mL) for 6 h and were stained with anti-human CD3-APC (eBioscience), anti-human CD4-PerCP (BD Bioscience, San Jose, CA, USA), anti-human CD25-FITC (eBioscience), and anti-human CD127-PE (eBioscience) for 20 min in dark at 4 °C. The labeled samples were read using a FACS Calibur analyzer (BD Bioscience) with CellQuest Pro software (BD Bioscience), and the data were analyzed using FlowJo version 7.6.2 for Windows (Tree Star Inc., Ashland, OR, USA). Isotype control antibodies were used to delineate positive and negative cells in the APC, PerCP, FITC, and PE fluorescence channels.

2.9. Real-time reverse quantitative PCR analysis

Total RNA was isolated from cultured PBMCs using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. cDNA was synthesized with random hexamers using PrimeScript RT Master Mix (TaKaRa, Dalian, Liaoning Province, China). Real-time