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Immune response of Th17-associated cytokines by peripheral blood mononuclear cells from patients with chronic hepatitis C virus infection

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ABSTRACT

Hepatitis C virus (HCV) chronic infection causes severe cellular immune dysfunction. Here, we investigated the production of Th17-associated cytokines by peripheral blood mononuclear cells (PBMCs) of untreated patients with HCV, patients presenting an early virologic response (EVR) after 12 weeks of treatment with interferon-α plus ribavirin with or without HCV protease inhibitors, and patients who were nonresponders to HCV therapy. PBMCs were stimulated with HCV core and nonstructural antigens, and the production of Th17-associated cytokines was measured with a Milliplex MAP immunoassay. Core-stimulated PBMCs from both untreated and nonresponder patients produced interleukin (IL)-17A, and vigorous production of IL-17A in response to NS3 antigen was only verified in the untreated group. Nonresponder patients also produced IL-17F after core antigen stimulation. IL-21 production was unaltered in the three groups of patients, whereas IL-17E and IL-22 were not detected. The production of Th17 cytokines by cells from patients showing an EVR was insignificant. IL-17A and IL-17F levels were not correlated with alanine aminotransferase levels or viremia. However, advanced fibrosis was associated with higher IL-17A production in T0 cells stimulated with core antigen. Untreated patients with HCV and patients who were nonresponders to antiviral treatment differed in their PBMC immune responses of Th17-associated cytokines. The early virological response to antiviral treatment dramatically decreased Th17 immune responses to HCV antigens.

1. Introduction

Chronic HCV infection is epidemic and associated with high morbidity and mortality. Although the global prevalence of HCV-seropositive individuals has been estimated at 1.6% (range: 1.3–2.1%), corresponding to 115 million people, the global prevalence of HCV-viremic persons has been estimated at 1%, corresponding to approximately 71 million [1].

Chronic HCV infection is associated with liver carcinoma; however, its persistence is only partially explained. Individuals who show control of acute HCV infection typically express interferon (IFN)-γ in the liver several weeks after the onset of the disease and produce chemokines and cytokines involved in antigen processing and presentation [2,3]. In these individuals, there is increased production of $CD4^+$ and $CD8^+$ T lymphocytes that specifically target epitopes of the core and nonstructural proteins (NS3/4A and NS5B) [4–6].

Th17 and other cells have been implicated in the adaptive immune response observed in chronic hepatitis C and other liver diseases, and some of their immune mediators, such as IL-17A, could be associated with liver damage and disease progression. Additionally, the degree of hepatic inflammation is directly correlated with the infiltration of Th17 cells [7–11].

The involvement of Th17-associated cytokines in antiviral treatments for chronic hepatitis C has rarely been investigated [12]. Thus, in this study, we investigated the production of Th17-associated cytokines by core- and nonstructural antigen-stimulated PBMCs from patients with HCV, including untreated patients, treated patients exhibiting an early virological response (EVR), and nonresponders to antiviral therapy.

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2. Materials and methods

2.1. Patients

Eighteen patients with chronic HCV infection (eight women and 10 men; mean age: 53 ± 9 years, range: 42–66 years) were enrolled in this study from August 2013 to November 2014. All were re-evaluated after 12 weeks of antiviral treatment. Eleven of 18 patients were only treated with IFN-α/ribavirin (PEG-IFNα2a 180 μg/week or PEG-IFNα2b 1.5 μg/kg/week combined with 1000–12,000 mg ribavirin). Seven of 18 were treated with PEGylated IFN-α/ribavirin plus Boceprevir (Victrelis; Merck Sharp & Dohme; 800 mg three times/day, 24–48 weeks) or PEGylated IFN-α/ribavirin plus Telaprevir (Incivo; Jansse-Cilag Farmacêutica Ltda; 750 mg three times/day, 12 weeks). The nonresponder (NR) group of patients was represented by 10 patients with a mean age of 55 ± 8 years (range: 42–66 years; four men and six women). These patients were previously treated with PEG-IFN-α plus ribavirin but exhibited significant viremia 24 weeks after treatment or did not show decreased HCV-RNA lower than 2 log IU/mL after 12 weeks of treatment.

The patients were diagnosed with chronic hepatitis C at the Gastro-Hepatology Service of the Professor Edgard Santos Hospital (Salvador, Bahia) after clinical, serological (third generation enzyme-linked immunosorbent assay [ELISA]), and molecular (HCV-RNA) exams. All patients in this study were infected with HCV genotype 1, and their data for viral load and liver histology or Fibroscan were obtained from their medical files. Patients with viral coinfections by human immunodeficiency virus, human T-lymphotropic virus, or hepatitis B virus or having neoplasia or chronic autoimmune or inflammatory diseases were not included in this study. All patients signed written consent to participate in the study, and the study was approved by the Ethics Committee of the Professor Edgar Santos Hospital.

2.2. Biochemical and immunological exams

Serum samples from patients were analyzed for alanine aminotransferase (ALT) levels the kinetic-UV method and for non-organ-specific autoantibodies (NOSAs) and cryoglobulins. Antinuclear antibodies (ANAs) were screened with an indirect fluorescent antibody test (IFAT) using HEp-2000 cells as a substrate (Immuno Concepts, USA; cutoff < 40). Anti-smooth muscle actin (SMA) antibodies (cutoff < 40) were also tested by IFAT on tissue sections from the kidney, liver, and stomach (Viro-Immun Labor-Diagnostika GmbH, Germany). Serum rheumatoid factor (RF) was evaluated using automated nephelometry (Image, Beckman Coulter, USA; cutoff < 20 IU/mL). Anti-CCP antibodies were investigated by indirect ELISA using citrullinated peptide antigens (ORGENTEC Diagnostika GmbH, Germany; cutoff < 20 U/mL). The presence of serum cryoglobulins was examined at 4 °C for 7 days, and their presence was confirmed by dissolution after 1 h of incubation at 37 °C.

2.3. Antigen stimulation of peripheral blood mononuclear cells (PBMCs)

PBMCs were obtained by centrifugation using a Ficoll-Paque Premium density gradient (GE Healthcare, Sweden). Cells were adjusted to 1.0×10^6 cells/mL in RPMI 1640 medium containing 10% fetal bovine serum, 2 mM glutamine, 25 mM HEPES (pH 7.2), and antibiotics (Cultilab, Brazil). Next, 10 mg/mL gentamicin (Sigma-Aldrich, USA) was added to this medium, and 100 μL of the cell suspension was added to the wells of a culture microplate (KASVI, China). The positive control was 10^5 cells/well stimulated with 10 μL of a phytohemagglutinin solution (PHA, 5 μg/mL), and the negative control was 10^5 cells/well cultured without antigen, but with 10 μL of the antigen diluent. Antigen tests were performed by adding 10 μL of each antigen solution containing 10 μg/mL of HCV recombinant core peptide (amino acids 2–192) and fragments of the HCV nonstructural proteins NS3 (amino acids 1450–1643), NS4 (amino acids 1658–1863), and NS5 (amino acids 2061–2302; Abcam, UK) to separate wells (containing 10^5 cells/well). The controls and tests were carried out in triplicate. The microplates containing PBMCs were incubated for five days at 37 °C in the presence of 5% CO_2. After incubation, the culture supernatants were obtained by centrifugation at 4 °C (1900 rpm for 10 min) and stored at −80 °C to determine cytokine levels.

2.4. Determination of cytokine levels

A multiplex immunoassay from Millipore (Milliplex MAP; Millipore Corporation, USA) was used to determine the levels of Th17-associated cytokines (interleukin [IL]-17A, IL-17F, IL-21, IL-22, and IL-17E) in culture supernatants of PBMCs (control and antigen-stimulated cells). The immunoassay was performed in an MAGPIX analyzer (Merck, USA), as recommended by the manufacturer. Cytokine levels were obtained using specific standard curves with MAGPIX × PONENT software (Millipore). The lower detection limits of this immunoassay were 2.1 pg/mL for IL-17A, 9 pg/mL for IL-17F, 9.9 pg/mL for IL-17E, 2 pg/mL for IL-21, and 2.1 pg/mL for IL-22.

2.5. Statistical analysis

Statistical analyses were performed using Prism version 6.0 (GraphPad Software Inc., USA). D'Agostino-Pearson tests were used to analyze the distributions of variables. Fisher's exact tests were used to investigate the associations between two certain groups. The medians of two groups were compared with nonparametric Mann-Whitney tests, and Wilcoxon tests were used to compare paired variables from a group. Kruskal-Wallis tests followed by Dunn's multiple comparisons tests were used to compare the medians of three or more groups. Chi-square tests were used to compare proportions. In the analyses, a p-value of less 0.05 was significant.

3. Results

3.1. Laboratory findings

This study involved 18 patients infected with HCV gen1 before and after 12 weeks of antiviral treatment. Fourteen untreated patients underwent liver histology following liver biopsy, and two were diagnosed with fibrosis using Fibroscan. Two individuals from this group were not evaluated for liver injury. Most untreated patients (11/16, 69%) had mild fibrosis (F1 and F2), while more advanced fibrosis (F3 and F4) was observed in five out of 16 (31%) patients. The presence of liver necro-inflammatory activity was examined in 14 patients. In two of these patients, necro-inflammatory activity was absent (A0, 14%). Three out of 14 (21%) patients showed slight activity, whereas seven of 14 exhibited mild necro-inflammatory activity (A2). Most patients (13/18, 72%) had a high viral load (> 800,000 IU/mL), but four patients had a low viral load (< 800,000 IU/mL). One patient did not undergo examination of viral load. Eight out of 18 (44%) untreated patients exhibited increased ALT activity (> 41 U/L). Antinuclear and anti-SMA antibodies were observed in only one (6%) and 8 (44%) of these patients, respectively, whereas the presence of RF was detected in seven of 18 (39%) patients. Anti-CCP antibodies were not found. Five of 18 patients (28%) had cryoglobulinemia.

After 12 weeks of treatment, 15 of 18 patients exhibited an EVR. Ten of 15 (67%) patients had undetectable viremia, and five (33%) presented a higher than 2-log reduction in their viral load of HCV. Three of 18 (17%) patients were null responders. The prevalence of NOSA (ANA, SMA, and RF) in the patients before and after antiviral treatment was similar.

The NR group of 10 patients presented a high viral load. Mild fibrosis (F2) was observed in five of 10 (50%) patients, and the other patients had an advanced stage of fibrosis (F3–F4). Five patients in this

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