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Role and recruitment of Th9 cells in liver cirrhosis patients

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ABSTRACT

Objective: To investigate the role of T helper 9 (Th9) cells in liver cirrhosis (LC) patients and whether chemokine receptor type 6 (CCR6)/chemokine ligand 20 (CCL20) axis is involving in the recruitment of Th9 cells into liver.

Methods: Peripheral blood and liver tissue from 30 LC patients and 18 normal controls were recruited. The frequency of Th9 cells and CCR4, CCR6 in the peripheral blood was tested by flow cytometry. Serum interleukin (IL)-9 and CCL20 levels were tested by enzyme-linked immunosorbent assay. Immunohistochemical staining was used to detect α-smooth muscle actin, CCR6 and CCL20 expression in liver tissue.

Results: The frequency of Th9 cells in LC patients was significantly increased compared with controls ($P < 0.05$). The serum IL-9 level and CCL20 level increased markedly in LC patients compared with controls ($P < 0.05$), and IL-9 was positively correlated to Th9 cells and CCL20. Furthermore, the frequency of Th9 cells was correlated to prothrombin time, total bilirubin level, hyaluronic acid and type IV collagen in LC patients. We also found that Th9 cells in LC patients expressed higher frequency of $CCR4^+$, $CCR6^+$ ($P < 0.05$). Compared with normal controls, the expression of CCR6 and CCL20 in LC tissue were significantly elevated ($P < 0.05$). The expression of α-smooth muscle actin was correlated to the CCR6 and CCL20 in liver tissue of LC patients.

Conclusions: This study suggests that Th9 cells may participate in the pathogenesis of LC, and the recruitment of Th9 cells into liver tissue might be through CCL20/CCR6 axis.

1. Introduction

Liver cirrhosis (LC) is the end stage of chronic liver injury, and most commonly caused by viral infections, alcoholism and fatty liver disease [1,2]. Like the spleen, the liver is one of the important immune organs, to which many immune cells are recruited under both physiological and pathological conditions [3]. Accumulating data have indicated the key role of infiltrating $CD4^+$ T cells in the progression of liver inflammation and fibrosis [4,5]. Currently, $CD4^+$ T helper (Th) cells can be subdivided into several major subsets, such as Th1, Th2, Th17 and regulatory T cells (Tregs), which have been reported to be involving in the pathogenesis of liver diseases [6].

Recently, Th9 cells have gained renewed interest because of their prominent roles in the regulation of host responses via interacting with the previously Th subsets. Th9 cells characteristically produce interleukin (IL)-9, which exerts pro-inflammatory or anti-inflammatory activities by modulating Treg and/or Th17 cell development and function [7–9]. Th9/IL-9 appears to function in a broad spectrum of autoimmune diseases and allergic inflammation. In addition, IL-9 also promotes the maintenance of tolerant environment by enhancing both Tregs and mast cell-mediated immunosuppressive functions [10–12]. To date, IL-9 has been implicated in some fibrosis diseases, such as pulmonary fibrosis and cystic fibrosis [13,14]. However, the role of IL-9 in liver fibrosis or LC has not been reported. Furthermore, little is known about the role of Th9 cells in the pathogenesis of LC. Although chemokine receptor usage by naïve,

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The study protocol was performed according to the Helsinki declaration and approved by Review Board at Guangxi Medical University for human studies. Informed written consent was obtained from each participant.

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Th1, Th2, Th17 and Treg cells is well documented [15,16], the migratory capacity of the functionally dynamic Th9 cells into liver tissue remains unknown. Therefore, in the present study, we evaluated the role of Th9/IL-9 in LC patients and determined the homing receptors involved in their recruitment to liver tissue.

2. Materials and methods

2.1. Patients and controls

The study protocol was approved by the Review Board at Guangxi Medical University for human studies, and written informed consent was obtained from each participant. All patients were either hospitalized or treated at the First Affiliated Hospital, Guangxi Medical University, between December 2013 and June 2014. Liver tissue and corresponding peripheral blood samples were collected from 30 LC patients with hepatitis B. Patients who were co-infected with HIV or other hepatitis viruses and autoimmune diseases were excluded. No one received anti-hepatitis B virus agents or steroids six months before sampling. Peripheral blood and liver tissue samples from 18 normal subjects were collected as controls. Patients with viral hepatitis, autoimmune hepatitis and alcoholic liver diseases were excluded from the normal controls.

2.2. Flow cytometry

Intracellular cytokines were studied by flow cytometry to reflex the cytokine-producing cells. The expression of markers on T cells from peripheral blood was determined by flow cytometry, as previously described [17]. After surface or intracellular staining with specific anti-human antibodies, the cells were conjugated with allophycocyanin, AlexaFluor 647, or phycoerythrin. These human antibodies included anti-CD4, anti-IL-9, and anti-IL-22 monoclonal antibodies, which were purchased from BD Biosciences (Franklin Lakes, NJ, USA). The T cell subsets were incubated in Roswell Park Memorial Institute1640 medium for 5 h at 37 °C in the presence of 5% CO_2 and stimulated with phorbol myristate acetate (50 ng/mL; Sigma–Aldrich, MO, USA), ionomycin (1 μg/mL; Sigma–Aldrich), and Golgi-Stop (1.7 μg/mL; BD Biosciences). Phorbol myristate acetate and ionomycin are pharmacological T-cell-activating agents that mimic signals generated by the T-cell receptor complex and have the advantage of stimulating T-cells of any antigen specificity. Monensin was used to block intracellular transport mechanisms, thereby leading to accumulation of cytokines in the cells [17,18]. After incubation, the cells were stained at room temperature in the dark for 30 min with allophycocyanin-conjugated anti-CD4 monoclonal antibodies. The cells were then stained with AlexaFluor 647-conjugated anti-IL-9 monoclonal antibodies and phycoerythrin-conjugated anti-IL-22 monoclonal antibodies (Cat. No. 560436) at 4 °C for 30 min after fixation and permeabilization. All the antibodies were from BD Bioscience PharMingen. Isotype controls were analyzed to ensure correct compensation and confirm antibody specificity. Stained cells were analyzed by flow cytometry using a FACScan Cytometer equipped with the CellQuest software (BD Bioscience PharMingen).

2.3. Immunohistochemistry

Immunohistochemical staining was performed by the streptavidin–biotin complex method according to the manufacturer's instructions. Paraffin-embedded, formalin-fixed liver tissues were cut into 4-μm sections and placed on polylysine-coated slides. Each paraffin section was deparaffinized and rehydrated through a graded series of ethanol. Endogenous peroxidase was blocked using a 3% H_2O_2 methanol solution. Goat serum albumin (5%; Zhongshan Golden Bridge Biotech, Beijing, China) was applied to block nonspecific staining. Primary antibodies [chemokine receptor type 6 (CCR6; Origene, USA), chemokine ligand 20 (CCL20; Bioss, Beijing, China) and α-smooth muscle actin (SMA; Sigma–Aldrich, USA)] were added and then incubated overnight at 4 °C. After washing with phosphate buffer saline (PBS) three times, incubation of biotinylated secondary antibodies was performed at room temperature. Slides were treated with streptococcus avidin–peroxidase and placed in the incubator at 37 °C for 30 min after washing with PBS three times. The slides were visualized by light microscopy after diaminobenzidine reaction. Pieces were sealed with neutral gum after counterstaining with hematoxylin. Positive tissue sections were used as a positive control and PBS was taken as a negative control in place of primary antibodies. Ten high-power fields of view were randomly selected for each slides. Image analysis of graphics was measured by Image Pro-Plus 6.0 software.

2.4. Measurement of cytokines and chemokine

The concentrations of cytokines IL-9 as well as chemokine CCL20 in serum were measured by ELISA kits according to the manufacturer's protocols (all kits were purchased from R&D Systems, Minneapolis, USA). All samples were assayed in duplicate.

2.5. Statistical analysis

Data are expressed as the mean ± SD. The statistical significance of Th9, IL-9, and CCL20 among the subjects was determined by Student's *t*-test or Wilcoxon's rank sum test where appropriated. The Pearson correlation test was used for correlation analysis depending on the data distribution. Analysis was completed with SPSS version 16.0 by Statistical Software (Chicago, IL, USA), and P value < 0.05 were considered to be statistically significant.

3. Results

3.1. Clinical data of the subjects

The demographic and clinical information of the LC patients and the normal controls are summarized in Table 1. The data showed no significant difference between cirrhotic patients and normal controls in term of age and gender. However, the liver function indexes of LC patients were deteriorated compared with controls.

3.2. Comparison of Th9 cells frequency in LC and normal control patients

We performed flow cytometry on mononuclear cells from blood to identify Th9 cells (Figure 1A–F). Th9 cells were

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