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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

Table 1
Clinical characteristics of subjects.

Clinical data	Normal controls (n = 18)	LC patients (n = 30)
Age (years)	42.6 ± 15.5	43.7 ± 16.4
Gender (M/F)	10/8	21/19
ALT (IU/L)	23.5 ± 12.7	58.6 ± 43.5*
AST (IU/L)	21.8 ± 10.6	82.6 ± 53.2*
TBIL (μmol/L)	12.6 ± 7.3	75.2 ± 17.7*
PT (s)	6.2 ± 3.5	18.1 ± 7.6*
ALB (g/L)	37.8 ± 18.9	28.6 ± 6.1*
HA (ng/mL)	65.7 ± 13.4	278.6 ± 168.7*
IV-C (μg/mL)	46.6 ± 15.2	233.1 ± 107.8*
PCIII (μg/mL)	47.8 ± 16.8	199.5 ± 82.7*
LN (μg/mL)	52.4 ± 18.5	197.5 ± 124.3*

*: $P < 0.05$ compared with normal controls. ALB: Albumin; TBIL: Total bilirubin level; PT: Prothrombin time; HA: Hyaluronic acid; IV-C: Type IV collagen; PCIII: Type III procolagen; LN: Laminin.

defined as $CD4^+$ $IL\text{-}9^+$ $IL\text{-}22^-$ cells. As shown in Figure 1G, the frequency of Th9 cells represented the higher values in LC patients compared with controls (3.55% ± 1.69% *vs.* 0.97% ± 0.24%, $P < 0.05$).

3.3. Serum IL-9 and CCL20 levels in LC patients

The ELISA test showed that, compared with controls, significant elevation of serum IL-9 and CCL20 levels were observed in the LC patients [IL-9: (25.55 ± 4.62) *vs.* (16.18 ± 1.32) pg/mL, $P < 0.05$; CCL20: (224.77 ± 33.73) *vs.* (34.58 ± 3.72) pg/mL, $P < 0.05$].

Correlation analysis revealed that the frequency of Th9 cells was positively correlated to the serum IL-9 levels in LC patients ($r = 0.413$, $P = 0.023$) (Figure 1H). The similar result was found between serum IL-9 and CCL20 ($r = 0.417$, $P = 0.015$). However, no significant correlation was found between Th9 cells and IL-9, and between IL-9 and CCL20 in normal controls ($P > 0.05$).

3.4. Expression of chemokine receptors on Th9 cells in the peripheral blood

The results of flow cytometry revealed that, most Th9 cells expressed high levels of CCR4 (85.8% ± 12.5% *vs.* 56.4% ± 2.36%, $P < 0.05$), and CCR6 (78.1% ± 10.6% *vs.* 56.4% ± 9.04%, $P < 0.05$) compared with controls.

Compared with controls, the expression of CCR6 and CCL20 in liver tissue of LC were significantly increased (CCR6: 0.027 ± 0.021 *vs.* 0.006 ± 0.001, $P < 0.05$; CCL20: 0.033 ± 0.011 *vs.* 0.017 ± 0.006, $P < 0.05$). In addition, a significant increased expression of α-SMA was detected in the LC patients compared to controls (0.054 ± 0.018 *vs.* 0.007 ± 0.001, $P < 0.05$). Moreover, we also observed that α-SMA was positively related to the CCR6, CCL20 in the liver tissue of LC ($r = 0.492$, 0.516, $P < 0.05$).

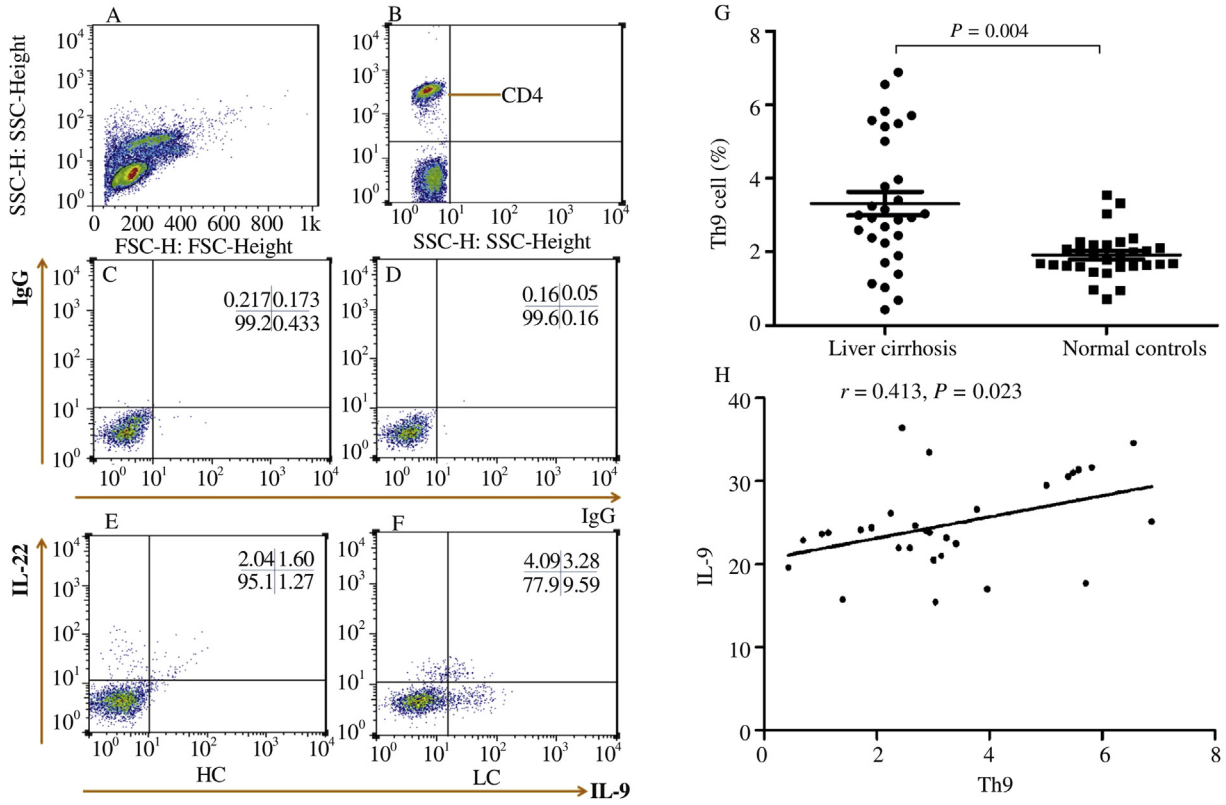

Figure 1. Representative flow cytometry dot plots demonstrate each group.
A: Lymphocytes were gated by flow cytometry; B: $CD4^+$ T lymphocytes were gated by flow cytometry; C–F: The representative flow cytometric dot-plots of isotype control in healthy control (HC) and LC group; G: Comparison of Th9 cells frequency between LC patients and HC. Horizontal bars indicate mean ± SD. Comparisons were made using a Wilcoxon signed-rank test; H: Correlation of Th9 cells frequency and serum IL-9 levels. Correlations were determined by Spearman rank correlation coefficients.

3.5. Expression of CCR6, CCL20 and α-SMA in liver tissue

CCR6 and its ligand, CCL20, have been reported with highly expression in a variety of human diseases [19,20]. The immunohistochemical staining images showed that CCR6 was expressed in the membrane of infiltration of lymphocytes in the portal area, the expression of CCL20 in the cell membrane of dendritic cells, hepatic stellate cells, fibroblasts, endothelial cells and cytoplasm of epithelial cells of bile duct in the portal area (Figure 2).

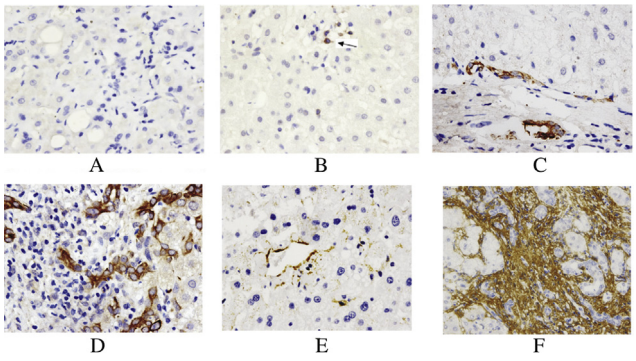

Figure 2. Immunohistochemical staining of CCR6, CCL20 and α-SMA in liver tissue.
A & B: Representative immunohistochemical staining of CCR6 in liver tissue of normal control and LC patients, respectively, the CCR6 was expressed in the cell membrane; C & D: Immunohistochemical staining of CCL220 in normal control group and cirrhosis liver tissue, respectively, the expression of CCL20 was in cell membrane and cytoplasm; E & F: Representative immunohistochemical staining of α-SMA in normal control and cirrhosis liver tissue, the expression of α-SMA was in portal area and fiber interval area. Positive expression is brown color (400×).

3.6. Correlation analysis between Th9 cells and liver function

Correlation analysis showed that the frequency of Th9 cells had a positive correlation with prothrombin time (PT), band total bilirubin level (TBIL) in LC patients ($P < 0.05$); the similar correlation also observed Th9 cells and hyaluronic acid (HA) and type IV collagen (IV-C) ($P < 0.05$) (Figure 3). However, no significant correlations were found between IL-9 and liver function and fibrosis indexes ($P > 0.05$).

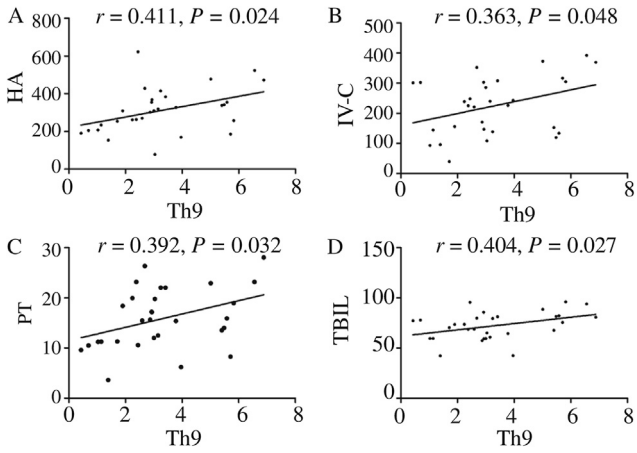

Figure 3. Correlation analysis between Th9 cells and liver function.
A: Correlation analysis between Th9 cells and HA; B: Correlation analysis between Th9 cells and IV-C; C: Correlation analysis between Th9 cells and PT; D: Correlation analysis between Th9 cells and TBIL.

4. Discussion

The role of Th9 cells have been implicated in many inflammatory and immune diseases. In allergic asthma patients, the Th9 cells frequency in peripheral blood was increased compared with allergic rhinitis and control subjects, the same tendency was observed for IL-9, and the serum IL-9 levels was correlated with frequency of Th9 cells [21]. In the context of experimental autoimmune encephalomyelitis (EAE), adoptive transfer of *in vitro*-generated encephalitogenic Th9 cells into naïve hosts led to development of neuroinflammatory central nervous system lesions and induction of severe EAE [22]; while inhibition of IL-9, using IL-9-deficient mice or IL-9 antibody, has been shown to alleviated EAE [23,24]. Collectively, these studies suggest that Th9 cells can contribute to the pathogenesis of autoimmunity in multiple organ systems.

In our study, we observed that the frequency of Th9 cells and serum IL-9 levels was significantly elevated in LC patients compared with that of normal controls. We also found that frequency of Th9 cells was positively related to the PT, TBIL, HA, IV-C in LC patients, suggesting that Th9 cells participated in the pathogenesis of LC. However, we did not observe significant correlation between IL-9 and liver function indexes, suggesting that the role of Th9 cells in LC can not equal to that of IL-9. The reasons of insignificant correlation is not known, we speculated that there were following reasons: first, besides Th9 cells, other Th cells, such as Th2, Th17 and Treg cells can also produce IL-9; second, the small sample size lead to a relatively statistical power.

Migration of Th cells to the lesion sites is essential for the execution of their effector function, where the chemokines and their receptors are orchestrating this process. Chemokines are a family of low molecular weight pro-inflammatory cytokines, which bind to chemokine receptors and sustain the migration of neutrophils, lymphocytes, monocytes and dendritic cells. Currently, the role of CCR6 and its ligand CCL20 is well documented in several diseases, including inflammatory bowel disease [20] and liver cancer [19]. In malignant pleural effusion, there was study reported that Th9 recruited to the pleural space during pleural metastasis by pleural mesothelial cells via a CCR6/CCL20 axis [25]. The CCR6/CCL20 axis mediates the migration of circulating Tregs into tumor microenvironment, which in turn results in tumor progression and poor prognosis in liver cancer patients [19].

Although Th9 cells have been implicated in both allergy and autoimmunity, the migratory properties of Th9 cells remain enigmatic. In the context of LC, migration of Th9 cells into the liver is important to the development and progression. In this study, we found that Th9 cells expressed a high level of CCR6 and CCR4 on their surface compared with normal controls. Because an increase of α-SMA was indication of severe LC, the correlation of CCR6, CCL20 and α-SMA in liver tissue suggested that CCR6 and CCL20 may participate in the pathogenesis of LC. In addition, the increased expression of CCR6 and CCL20 in serum also further confirmed this role. Taken together, these data suggested that the CCR6/CCL20 axis might be related to the infiltration of Th9 cells into liver tissue. The mechanism of CCR6/CCL20 axis mediating Th9 cells to the liver remains to be elucidated. According to the previous report,

we speculated that IL-9 can induce the expression of CCL20 within the lesions site and CCR6 expression on leukocytes to facilitate tumor infiltration [26]. However, a chemotaxis assay is necessary in order to further confirmed this role.

The present study gives a preliminary insight into the role of Th9 cells in the pathogenesis of LC, and suggests that the CCR6/CCL20 axis might recruited Th9 cells into liver tissue to execute their effector function. However, some limitations should be noted. First, we observed the role of Th9 cells in LC, but the mechanism of this role remains to be elucidated, especially how the Th9 cells act this role via IL-9 and the interaction between other IL-9 producing Th cells. Second, in this study, we only investigated CCR6/CCL20 axis, other chemokine axis, such as chemokine receptor-4/Stromal cell-derived factor 1 [27], CCL21/CCR7 [28], which have been reported in several immune diseases, were not investigated in our study. Thus, these chemokine axis should be investigated in further study. Third, the number of included subjects was small, which may be the reasons of insignificant correlation between Th9 cells and other indexes. Therefore, in order to enhance the statistical power a larger sample size is need in further study.

In conclusion, the present study suggests that Th9 cells participate in the pathogenesis of LC, and the recruitment Th9 cells into liver tissue might be through CCR6/CCL20 chemokine axis.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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