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Increased IL-10/IL-17 ratio is aggravated along with the prognosis of patients with chronic lymphocytic leukemia

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ABSTRACT

Objective: This study is to investigate the association between the Treg/Th17 cells and prognosis of chronic lymphocytic leukemia (CLL).
Methods: Totally 50 CLL patients and 20 Health controls were included in this study. Regulatory T (Treg) cells and the cell subset secreting IL-17 (Th17) in peripheral blood were detected with flow cytometry. Serum levels of IL-10 and IL-17 were determined with ELISA, and expression of Foxp3 and RORγt was assessed with quantitative real-time PCR.
Results: Treg and Th17 cell proportions in peripheral blood in the CLL patients were significantly higher than control. Serum levels of IL-10 and IL-17, and expression of Foxp3 and RORγt, were significantly increased in the CLL patients. Ratios of Treg/Th17 and IL-10/IL-17 were significantly elevated in the CLL patients. Compared with those before treatment, Treg/Th17 and IL-10/IL-17 ratios were declined in the CLL patients in remission. Compared with the non-remission group, Treg cells were significantly decreased, while Th17 cells were significantly increased, resulting in decreased Treg/Th17 ratio, in the remission group. Moreover, the serum IL-10 level was significantly decreased, while the serum IL-17 level was significantly increased, resulting in declined IL-10/IL-17 ratio, in the remission group. Correlation analysis showed that, Treg and Th17 cell counts were significantly associated with CD38 and ZAP-70 expression in the CLL patients. Moreover, the IL-10/IL-17 ratio was also significantly associated with CLL prognostic factors.
Conclusion: Altered Treg/Th17 and IL-10/IL-17 ratios in CLL would be aggravated along with the disease progression, which might be used as indicators for the disease prognosis.

1. Introduction

Chronic lymphocytic leukemia (CLL) is a type of cancer that involves the excessive proliferation of B lymphocytes, which is one of the most common leukemia forms (incidence accounting for 25%–40%) [1–3]. Pathogenesis of CLL has been related to the immune abnormalities [4, 5]. Concerning the immune function, regulatory T (Treg) cells participate in the anti-inflammatory responses and the immune tolerance maintenance. Forkhead box protein p3 (Foxp3) is a Treg cell-specific transcription factor, which mainly regulate the development and function of $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells. It has been shown that the excessive proliferation of $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells contributes to the tumor growth in patients with ovarian cancer, and Treg cells are negatively associated with the patient survival rates [6]. Moreover, in patients with hepatocellular cancer (HCC), $CD4^+$ $CD25^+$ Treg cells are increased in the peripheral blood, ascites, and tumor tissues, implying that these cells may suppress the anti-tumor immune responses [7].

It has been well accepted that, Treg cells could inhibit the effector cell function and suppress the immune responses, via secreting inhibitory cytokines such as IL-10 and TGF-β [8]. In recent years, a new T cell subset secreting IL-17 (called Th17 cells) has been shown to be able to induce inflammation. IL-17 can promote the release of pro-inflammatory cytokines to amplify the inflammatory response, and its binding to the receptor would mobilize the neutrophils to mediate the tissue inflammation. The proportion of Th17 out of Treg cells, and the balance between IL-10 and IL-17, may affect the precise modulation of immune responses. Treg cells have been found to increase along with

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the CLL progression, inhibiting the anti-tumor immune responses [9–13]. The increase in total Treg cells would influence the Treg/Th17 balance, and the disturbed balance would promote the disease progression in turn [14]. However, the association between the disturbed Treg/Th17 balance and disease prognosis has not yet been clearly elucidated.

In this study, the associations between the Treg/Th17 and IL-10/IL-17 ratios with the prognosis of CLL were investigated. Treg and Th17 cells were detected with flow cytometry in the CLL patients, before and after treatment. The serum levels of IL-10 and IL-17, as well as the mRNA expression levels of Foxp3 and RORγt (a transcription factor regulating Th17 cell differentiation), were also detected. After treatment, the Treg/Th17 and IL-10/IL-17 ratios were analyzed and compared between the remission and non-remission groups of CLL patients. The relevance with the disease prognosis and the clinical significance were discussed.

2. Materials and methods

2.1. Study subjects

Totally 50 patients with CLL, 32 males and 18 females, with an average age of 67.3 ± 8.71 years, were included in this study, who were admitted to the First Affiliated Hospital of Xinjiang Medical University, from January 2013 to December 2014. Another 20 healthy subjects, 15 males and 5 females, with an average age of 62.5 ± 12.9 years, without autoimmune diseases, cancers, or recent infection, were used as control. Basic information of these subjects and clinical data at admission were collected in Table 1. Moreover, the results of disease-related clinical detection were shown in Table 2. Prior written and informed consent were obtained from every patient and the study was approved by the ethics review board of the First Affiliated Hospital of Xinjiang Medical University.

2.2. Drug treatments

These CLL patients were treated according to the iwCLL treatment guideline [15]. In these 50 patients, 25 were subjected to the FC remedy of fludarabine and cyclophosphamide; 6 were subjected to the FCR remedy of fludarabine, cyclophosphamide, and rituximab; and 19 were treated with chlorambucil alone. After treatments, according to the therapeutic effects, these 50 patients were divided into the remission (complete remission, CR and partial remission, PR; n = 36) and non-remission (progressed disease, PD and stable disease, SD; n = 14) groups.

2.3. Detection of $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells

Peripheral blood (4 mL) was collected from each subject under fasting condition. Mononuclear cells were separated with Ficoll, and the cell density was adjusted to 2×10^6 cells/mL. 100 μL cell suspension was incubated with 5 μL CD3-Percp, CD4-FITC, and CD25-APC antibody (eBioscience, San Diego, CA, USA) in dark at 4 °C for 30 min. Then 1 mL 1× fixing buffer was added, and the cell suspension was kept in dark at 4 °C for 30 min. After adding 80 μL penetrating buffer, the cells were incubated with 5 μL PE-Foxp3 (eBioscience) or PE-IgG2a antibody (eBioscience) in dark at 4 °C for 30 min. The fluorescence was detected with an Aria II flow cytometer (BD, San Jose, CA, USA). Lymphocyte populations were selected on the FSC/SSC scatter plot. Fig. 2A shows the gating criteria to define the $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cell population in all the samples. Treg cells were given as a percentage out of the total $CD4^+$ T cells.

Table 1
Basic information and clinical data at admission for CLL patients and normal subjects.

	CLL patients	Normal subjects
Sex, male/female	32/18	15/5
Age, years	67.3 ± 8.71	62.5 ± 12.9
White blood cells, $\times 10^9$/L	31.02 ± 16.23	4.52 ± 0.88
Lymphocytes, $\times 10^9$/L	25.13 ± 12.65	1.49 ± 0.52
Lymphocyte percentage, %	80.98 ± 8.75	27.35 ± 6.21
Hemoglobin, g/L	122.34 ± 28.13	125.16 ± 4.36
Platelets, $\times 10^9$/L	141.92 ± 54.46	176.23 ± 40.25
Lymphadenopathy rate, % (n)	82.00% (41/50)	–
Splenomegaly rate, % (n)	63.25% (32/50)	–
β2-Microglobulin, mg/L	3.79 ± 2.01	2.59 ± 0.36

Table 2
Clinical characteristics of CLL patients.

	n
Binet staging (A/B/C)	15/16/19
Rai staging (0–II/III–IV)	22/28
ZAP-70 expression (positive/negative)	24/26
IgVH expression (positive/negative)	27/23
CD38 expression (positive/negative)	22/28
FISH	
Normal	18
del (13q14)	13
del (11q22)	9
del (17p13)	5
del (13q14) (11q22)	2
del (17p13) (13q14)	3

2.4. Detection of Th17 cells

Mononuclear cell suspension was diluted with RPMI 1640 medium (1:1 v/v; Gibco, Grand Island, NY, USA), and treated with PMA, ionomycin, and monensin (Sigma-Aldrich, St. Louis, Missouri, USA) in a 37 °C, 5% CO_2 incubator for 4 h. 100 μL cell suspension was incubated with 5 μL CD3-Percp and CD4-FITC antibodies (eBioscience) in dark at 4 °C for 30 min. After adding 80 μL penetrating buffer, these cells were treated with 10 μL IL-17A-PE monoclonal antibody (eBioscience) or PE-IgG (eBioscience) in dark at 4 °C for 30 min. Fluorescence was detected with flow cytometry, and lymphocyte populations were selected on the FSC/SSC scatter plot. $CD4^+$ cells were selected based on the CD4/CD3 gating, and Th17 cells ($CD4^+$ $IL\text{-}17^+$) were analyzed, which were given as a percentage out of the total $CD4^+$ T cells.

2.5. Quantitative real-time PCR

Peripheral blood mononuclear cells were collected, and total RNA was extracted with TRIzol LS reagent (Invitrogen, Carlsbad, CA, USA). The cDNA was obtained with the kit from Thermo Scientific, San Jose, CA, USA. Quantitative real-time PCR was performed with the SYBR Select Master Mix (ABI, Grand Island, NY, USA), following the manufacturer's instructions. The primer sequences were as follows: Foxp3, forward 5′-GCAGCTCTCAACGGTGGAT-3′ and reverse 5′-GGGATTTGGGAAGGTGCAGA-3′; RORγt, forward 5′-GCCAAGGCTCAGTCATGAGA-3′ and reverse 5′-CCTCACAGGTGATAACCCCG-3′; and GAPDH, forward 5′-TGTTGCCATCAATGACCCCTT-3′ and reverse 5′-CTCCACGACGTACTCAGCG-3′. PCR amplification conditions consisted of 50 °C for 2 min, 95 °C for 2 min, 95 °C for 15 s, and 60 °C for 1 min, for totally 40 cycles. The $2^{-\Delta\Delta CT}$ method was used to calculate the gene expression.

2.6. Enzyme-linked immunosorbent assay (ELISA)

Peripheral blood sample was collected, and the serum was separated. Serum levels of IL-17 and IL-10 were detected with the ELISA kit (eBioscience, San Diego, CA, USA), according to the manufacturer's instructions.