Original Article
Relationship of regulatory T cell level in peripheral blood with curative efficacy and prognosis of patients with non-Hodgkin's lymphoma

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Abstract: Regulatory T cells (Treg) are a subgroup of T cells with immunosuppressive function that play critical roles in antitumor immune response and immune escape. This study investigated Treg cells expression in Non-Hodgkin's Lymphoma (NHL) to discuss its relationship with clinical characteristics, curative effect, and prognosis. B lymphocyte NHL patients diagnosed in our hospital between Jan 2014 and May 2015 were selected. Flow cytometry was used to test $CD4^+CD25^+Foxp3^+$ $T/CD4^+$ T cell ratio in peripheral blood before and after chemotherapy to analyze its relationship with clinical features. The patients were divided into two groups upon the median of Treg cell percentage to compare the chemotherapy effect and survival rate. Peripheral $CD4^+CD25^+Foxp3^+$ $T/CD4^+$ T cell ratio in NHL patients (8.35 ± 1.43%) was significantly higher than normal control (1.98 ± 0.44%) before chemotherapy. It obviously declined after chemotherapy (4.56 ± 1.11%) but was still higher than healthy control. Peripheral Treg cell ratio in NHL patients was significantly correlated with clinical stage ($r = 0.745$, $P = 0.041$) and IPI score ($r = 0.798$, $P = 0.030$). Treg cell proportion in patients with elevated lactic dehydrogenase (LDH) was markedly higher than the normal LDH group ($t = 2.488$, $P = 0.007$). The survival rate of high Treg group was apparently lower than the low Treg group ($\chi^2 = 4.719$, $P = 0.029$). Peripheral Treg ratio significantly increased NHL patients and was related to clinical stage and IPI. Treg elevation patients presented worse chemotherapy response, survival rate, and prognosis.

Keywords: Treg cell, non-Hodgkin's lymphoma, curative effect, prognosis

Introduction

Non-Hodgkin's lymphoma (NHL) is a kind of lymphatic hematopoietic system malignant tumor occurred in lymphoid organ including lymph nodes, spleen, and thymus and/or extra-nodal lymphatic tissue and organ [1, 2]. In recent years, NHL incidence increased year by year, accounting for the 7th among all malignant tumors [3]. NHL can be derived from the B cells, T cells, and NK/T cells. B cell NHL accounts for the vast majority of NHL at 70%~85% [4]. NHL is one of the most common blood lymphatic system malignant hyperplastic diseases that closely relates to various immune functions. It usually affects the growth and function of immune cells, belonging to the immune system malignant tumor. NHL patients often appear immune dysfunction, and cellular and humoral immune dysfunction. NHL is featured as high invasion, dissemination, immune tolerance,

and immune deficiency, leading to poor prognosis and high fatality rate. Sakaguchi et al. [5] in 1995 first found a type of regulatory T cells (Treg) subgroup expressed CD4 and CD25 and presented immunosuppressive function. Forkhead transcription facter3 (Foxp3), a kind of transcriptional regulation factor in foxhead family, specifically expresses in $CD4^+CD25^+$ Treg cells and plays a key function for Treg cell development and function maintenance, thus is considered as the characteristic marker for $CD4^+$ $CD25^+$ Treg cells identification [6]. $CD4^+CD25^+$ $Foxp3^+$ T cells exhibit suppression of multiple immune cells, including $CD4^+$ T cells, $CD8^+$ cytotoxic T cells, B cells, and antigen presenting cells (APC), inhibition of the immune reaction, and induction of immune tolerance. Except inflammatory disease and autoimmune disease, Treg cells are also related to downregulating antitumor immunity, triggering immune escape mechanism, and promoting malignant

Table 1. Peripheral Treg cell proportion in two groups

	Cases	Treg/CD4$^+$ T (%)
Healthy control	40	1.98 ± 0.44
NHL	128	8.35 ± 1.43*

*$P < 0.05$, compared with healthy control.

tumor cell proliferation [7, 8]. Treg cells induced cellular immune state changes may have an influence on treatment sensitivity [9] and tumor recurrence [10]. Following the application of cellular immune therapy in the field of in cancer therapy, detection of cellular immunity in tumor patients and investigation of its relationship with disease characteristics, clinical curative effect, and prognosis are of great significance. This study detected CD4$^+$CD25$^+$Foxp3$^+$ Treg cells expression in the peripheral blood of NHL patients, and explored its relationship with clinical characteristics, curative effect, and prognosis to provide new idea for NHL treatment and prognosis improvement.

Materials and methods

Clinical information

A total of 128 B cell NHL patients received treatment in Guizhou Provincial People's Hospital between Jan 2013 and May 2015 were enrolled, including 80 males and 48 females with the average age at 48.6 ± 9.2 (11~76) years old. There were 61 cases of diffuse large B cell lymphoma, 10 cases of follicular lymphoma, 12 cases of mantle cell lymphoma, and 45 cases of mucosa associated lymphoid tissue lymphoma. According to the Ann Arbor staging criteria published by American joint committee on cancer (AJCC) and union for international cancer control (UICC), there were 35 cases in stage I, 30 cases in stage II, 40 cases in stage III, and 23 cases in stage IV. According to NHL international prognostic index (IPI), there were 52 cases scored 0~1, 20 cases scored 2, 38 cases scored 3, and 18 cases scored 4~5. Another 40 healthy physical examinees were selected as control, including 26 males and 14 females with mean age at 45.6 ± 12.3 (19~69) years old. No statistical difference was observed on age and gender between two groups ($P > 0.05$).

This study has been pre-approved by the ethical committee of Guizhou Provincial People's Hospital. All subjects have signed the consent forms before recruitment in this study.

Treg cell detection

Fasting peripheral blood was extracted from all subjects with heparin anticoagulation. A total of 100 µl blood was moved to a flow tube and treated by 5 µl FITC-CD4, PE-CD25, and APC-Foxp3 antibodies (BD Pharmigen). The flow tube with corresponding isotype control was set as control and used to adjust the compensation (BD Pharmigen). The cells were incubated at room temperature away from light for 20 min, and then added with hemolysin at room temperature away from light for 20 min. Next, the cells were centrifuged at 1000 rpm for 5 min and washed by PBS. At last, the cells were resuspended in 0.5 ml PBS and detected by Beckman FC 500 MCL flow cytometry to analyze the proportion of CD4$^+$CD25$^+$Foxp3$^+$ T cells in CD4$^+$ T cells of peripheral blood.

Chemotherapy

CHOP, CHOP-E, or R-CHOP chemotherapy regimens were the first choice for all subjects. The chemotherapy sustained for at least 6 cycles, continued for 2 cycles after complete remission, or progress. According to the curative effect judgment standard of malignant lymphoma [11], the patients were divided into complete remission (CR), partial remission (PR), stable disease (SD), and progressive disease (PD).

Statistical analysis

SPSS 18.0 software was used for data entry and statistical analysis. Measurement data was presented as mean ± standard deviation and compared by one-way ANOVA or LSD test. Enumeration data was depicted as percentage and analyzed by chi-square test. Patients' survival curve was formulated by Kaplan-Meier method. A $P < 0.05$ was considered as statistical significance.

Results

Peripheral Treg cell proportion increased in NHL patients

The ratio of CD4$^+$CD25$^+$Foxp3$^+$ T/CD4$^+$ T cell in peripheral blood from NHL patients and healthy

Table 2. The relationship between peripheral Treg cell proportion and clinical characteristics in NHL patients

Clinical characteristics	Cases	Treg/CD4+ T (%)	t/F value	P value
Gender			1.247	0.108
Male	80	7.98 ± 1.82		
Female	48	8.39 ± 1.77		
Age			0.792	0.215
≤ 60 years old	53	8.13 ± 2.01		
> 60 years old	75	8.41 ± 1.94		
Pathological type			0.130	0.942
Diffuse large B cell lymphoma	61	8.25 ± 1.93		
Follicular lymphoma	10	7.98 ± 1.85		
Mantle cell lymphoma	12	8.38 ± 2.01		
Mucosa associated lymphoid tissue lymphoma	45	8.11 ± 1.79		
Clinical stage			2.993	0.034
Stage I	35	7.37 ± 1.13		
Stage II	30	7.94 ± 1.65		
Stage III	40	8.19 ± 1.87		
Stage IV	23	8.59 ± 1.71		
IPI score			3.265	0.024
0~1	52	7.41 ± 1.26		
2	20	7.92 ± 1.71		
3	38	8.25 ± 1.67		
4~5	18	8.46 ± 1.69		
LDH			2.488	0.007
Normal	57	7.67 ± 1.96		
Elevation	71	8.52 ± 1.89		

Table 3. Correlation analysis of Treg ratio with clinical stage and IPI

	Treg/CD4+ T	
	r	P
Clinical stage	0.745	0.041
IPI	0.798	0.030

control was listed in **Table 1**. It was showed that The ratio of CD4+CD25+Foxp3+ T/CD4+ T cell in NHL patients before chemotherapy was significantly higher than the healthy control (t = 44.151, P < 0.001).

Relationship between peripheral Treg cell proportion and clinical characteristics in NHL patients

The peripheral CD4+CD25+Foxp3+ T/CD4+ T cell proportion in NHL patients showed no statistical difference on gender, age, and pathological types (P > 0.05, **Table 2**). The peripheral

CD4+CD25+Foxp3+ T/CD4+ T cell ratio was different in NHL patients with different clinical stages and IPI scores. Patients with late stage and high IPI score exhibited higher Treg cell ratio than the patients in early stage and low IPI score. Treg cell proportion in patients with elevated lactic dehydrogenase (LDH) was markedly higher than the normal LDH group (t = 2.488, P = 0.007). Peripheral Treg cell ratio in NHL patients was significantly correlated with clinical stage (r = 0.745, P = 0.041) and IPI score (r = 0.798, P = 0.030) (**Table 3**).

Treg cell ratio affected the chemotherapy response on NHL patients

The ratio of CD4+CD25+Foxp3+ T/CD4+ T cell in peripheral blood from NHL patients before chemotherapy was obviously higher than the healthy control (P < 0.05). Peripheral CD4+ CD25+Foxp3+ T/CD4+ T cell proportion in NHL patients significantly decreased after chemo-

Table 4. Treg cell ratio comparison before and after chemotherapy

	Cases	Treg/CD4+ T (%)
Healthy control	40	1.98 ± 0.44
NHL		
Before chemotherapy	128	8.35 ± 1.43*
After chemotherapy	128	4.56 ± 1.11*,#

*$P < 0.05$, compared with healthy control. #$P < 0.05$, compared with before chemotherapy.

Table 5. Treg cell proportion comparison in NHL patients with different outcome before chemotherapy

	Cases	Treg/CD4+ T (%) 13.705
CR	25	6.87 ± 1.06
PR	41	7.86 ± 1.74*
SD+PD	46+16	9.15 ± 2.31*,#

*$P < 0.05$, compared with CR group. #$P < 0.05$, compared with PR group.

therapy ($P < 0.05$), but was still higher than the healthy control ($P < 0.05$) (**Table 4**).

NHL patients with different treatment outcome presented statistical different peripheral CD4+CD25+Foxp3+ T/CD4+ T cell proportion before chemotherapy ($P < 0.05$) (**Table 5**). Peripheral Treg/CD4+ T cell ratio in CR patients were obviously lower than in PR, SD, and PD patients. PR patients exhibited markedly lower Treg/CD4+ T cell proportion than SD and PD patients.

The patients were divided into two groups upon the median of Treg cell percentage. In low ratio group, 17 cases obtained CR (26.6%), 26 cases got PR (40.6%), and 21 cases exhibited SD and PD (32.8%) after chemotherapy. On the contrary, there were 8 cases of CR (12.5%), 15 cases of PR (23.4%), and 41 cases of SD and PD (64.1%) in high ratio group after chemotherapy ($\chi^2 = 12.643$, $P = 0.002$, **Table 6**).

Treg cell ratio affected the prognosis of NHL patients

All of the 128 NHL patients were followed up for 8.6~36.7 months to compare the survival rate. The two-year survival rate in CD4+CD25+Foxp3+ T/CD4+ T cell ratio in low ratio and high ratio groups were 82.9% and 69.2%, respectively. The three-year survival rate was 59.5% and 32.9%. Log-rank test showed that the survival curve between two groups was significantly different ($\chi^2 = 4.719$, $P = 0.029$, **Figure 1**).

Discussion

NHL is a type of malignant tumor occurred in immune cells, tissues, and organs. The patients often have immunodeficiency, cellular and humoral immune dysfunction [12, 13]. Immunodeficiency state conditions, such as HIV/AIDS and immunosuppressive therapy, can significantly increase the risk of NHL. Thus, it is generally believed that NHL attack is associated with autoimmune function and status [14]. Immune system plays an important role in cancer surveillance, thus normal cell malignant transformation can trigger a series of immune response and eliminate tumor cells upon various immune mechanisms which are mainly composed of cellular immunity to prevent cancer [15, 16]. In addition, immune surveillance also plays a key role in removing residual tumor cells and tumor stem cells to prevent tumor recurrence. The surface antigen on tumor cells is featured as weak immunogenicity and antigenic modulation. It can lead to tumor cells evade immune surveillance and attack when the effective immune response is suppressed by major histocompatibility complex class I molecules and costimulatory molecules downregulation, or antitumor immune responses inhibition, resulting in tumor genesis and recurrence. However, the mechanism of immune escape is still unclear. CD4+CD25+ Treg cell is a kind of T cell subgroup with the potential immune regulating function. It "actively" suppresses CD4+ T cell activation and proliferation mainly through secreting immunosuppressive factors, such as TGF-β and IL-10, to play a crucial role in immunosuppression, immune homeostasis maintenance, and autoimmune disease prevention [17]. In recent years, it was found that CD4+CD25+ Treg cell is one of the important mechanisms involved in tumor cells "immune escape" [18]. Treg cells elevation could be found in the peripheral blood of a variety of cancers [19]. Curiel et al. [20] found that Treg cells can inhibit the immune response to ovarian tumor, abate the antitumor immune effect caused by tumor vaccine, and worsen prognosis. Animal experiments showed that removal of Treg cells can obviously prolong the survival of bearing tumor animal, even induce tumor regression [21]. In addition to suppressing CD4+ T cells, Treg cells was also confirmed to weaken tumor immune response and promote tumor occurrence or recurrence by inhibiting CD8+ T cells [22], NK cells [23], and antigen

Table 6. The relationship between Treg cell ratio and chemotherapy response

$CD4^+CD25^+Foxp3^+$ T/$CD4^+$ T cell ratio (%)	CR (cases, %)	PR (cases, %)	SD+PD (cases, %)	χ^2	P
Low ratio group (3.42%~8.29%)	17 (26.6%)	26 (40.6%)	21 (32.8%)	12.643	0.002
High ratio group (8.29%~13.76%)	8 (12.5%)	15 (23.4%)	41 (64.1%)		
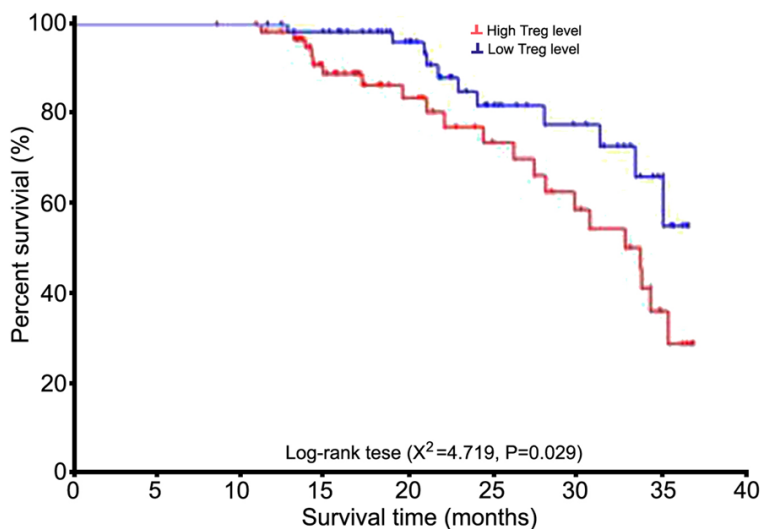
Figure 1. Treg cell ratio affected the prognosis of NHL patients.

presenting cell surface costimulatory molecules expression [24]. Sakaguchi, et al. [5] first isolated Treg cells expressed CD4 and CD25, and with immunosuppressive function. Another study demonstrated that activated functional T cells also expressed CD25 on the surface, thus the accuracy of CD4 and CD25 in labeling Treg cells was influenced. Foxp3 nuclear transcription factor, first reported by Brunkow in 2001 [25], is mainly expressed in a variety of lymphoid tissues and organs. Foxp3 expression and function is closely related to Treg cells by regulating $CD4^+CD25^+$ Treg cells differentiation and development. Foxp3 is the essential regulatory factor to maintain $CD4^+CD25^+$ Treg cells development and functional exertion. Many studies confirmed that Foxp3 was most accurate and specific marker for Treg cells [6, 26, 27].

Several studies demonstrated that Treg cells percentage increased in the peripheral blood of many kinds of cancers, such as lung cancer [28], liver cancer [29], colorectal cancer [30], and leukemia [31]. Moreover, it was correlated with clinical features, disease progression, and prognosis. The relationship between Treg cells expression in NHL patients and clinical features, treatment effect, and prognosis are still controversy. This study detected $CD4^+CD25^+Foxp3^+$ Treg cells expression in peripheral blood from NHL patients and discussed its relationship with clinical characteristics, curative effect, and prognosis. Compared with healthy control, peripheral $CD4^+CD25^+Foxp3^+$ T/$CD4^+$ T cell proportion significantly increased in NHL patients before chemotherapy, suggesting that Treg elevation may induce immune inhibition, leading to cancer cells evade immune surveillance and immune clearance, which was in accordance with Mittal results [32]. Han, et al. [33] found that B lymphocytes underwent malignant transformation in B cell NHL patients can induce $CD4^+CD25^-$ T cells transform to $CD4^+CD25^+Foxp3^+CD127^{low}$ Treg cells and participated in promoting immune escape and B cell NHL occurrence. Further analysis demonstrated that peripheral $CD4^+CD25^+Foxp3^+$ T/$CD4^+$ T cell percentage also presented obvious difference in different clinical stages, IPI scores, and LDH levels. Mittal, et al. [32] reported that Treg cell level was positively correlated with LDH level and clinical stage in treat-naïve NHL patients, which was similar to our results. LDH is recognized as an independent prognosis indicator for prognosis, as its elevation often reflects high malignant degree and large tumor load. This study observed that Treg cell ratio in patients with LDH elevation was obviously higher than that with normal LDH level, indicating that Treg cell proportion increase may participate in immunosuppressive action and enlarge tumor load in NHL patients. After chemotherapy, $CD4^+CD25^+Foxp3^+$ T/$CD4^+$ T cell percentage significantly reduced compared with before chemotherapy but higher than the healthy control. It may be caused by that immune function recovered after treatment, leading to part of antitu-

mor immune response activation, whereas the body is still in a state of immunosuppression. As a type of immunosuppressive cell, Treg cell percentage can affect the treatment effect and reflect the remission. This study revealed that patients with better curative effect (CR) showed obviously lower Treg cell percentage before treatment than the patients with worse effect (PR, SD, PD). On the contrary, patients with high Treg proportion obtained worse chemotherapy effect than patients with low Treg ratio. Survival analysis presented that the prognosis of patients with high Treg ratio was markedly worse than that of patients with low Treg percentage. Thus, peripheral Treg elevation induced immune suppression, which was closely associated with NHL occurrence. Monitoring Treg cells in NHL patients is of guiding significance to observe disease status and evaluate chemotherapy response and prognosis.

Conclusion

Peripheral Treg cell percentage obviously increased in NHL patients and related to clinical stage and IPI. Patients with Treg increase exhibit worse chemotherapy curative effect, survival rate, and prognosis.

Disclosure of conflict of interest

None.

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