·Clinical Research·

Correlation between peripheral blood $CD4^+CD25^{high}$ $CD127^{low}$ regulatory T cell and clinical characteristics of patients with non-Hodgkin's lymphoma

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[Abstract] Background and Objective: Non-Hodgkin's lymphoma (NHL) is a malignant disease originating from immune system. Studies of the possible relationship between NHL and immune suppression status are of great concern. Regulatory T cell (Treg) is a subtype of T cells that exert an immunosuppressive function. However, the relationship between Treg and lymphoma is controversial. The study was to detect peripheral blood levels of Treg in patients with NHL and healthy adults, and to explore the possible relationship between peripheral blood Treg level and NHL. **Methods:** By using flow cytometry with surface staining fluorochrome-conjugated antibodies for CD4, CD25, CD127, the percentages of $CD4^+CD25^{high}CD127^{low}$ Treg in peripheral blood of 31 healthy adults and 99 newly diagnosed NHL patients, hospitalized in Sun Yet-sen University Cancer Center from December 2006 to March 2008, were detected and analyzed. **Results:** The average peripheral blood $CD4^+CD25^{high}CD127^{low}$ Treg levels were 8.07±1.90 and 11.20±4.40 in healthy adults and newly diagnosed NHL patients, respectively. The difference of peripheral blood Treg levels between them was statistically significant (P<0.001,95% CI: 2.02–4.23). The peripheral blood level of Treg was significantly higher in the male NHL patients than in the female patients(P=0.030,95% CI: 0.19–3.77). Patients with bad habits (smoking, addict to drink, or both) had significantly higher peripheral blood Treg level than patients without bad habits (P=0.045,95%CI: 0.04–3.84). It was no significant relation between peripheral blood Treg level and age, stage, IPI, B symptom, bulky disease, LDH level, pathologic subtype, short term response, HBV infection, and so on. The analysis in diffuse large B-cell lymphoma (DLBCL) subtype showed the same results. **Conclusions:** Newly diagnosed NHL patients are in an immunosuppressive statue. Patients with bad habits (smoking, addict to drink, or both) have higher peripheral blood Treg level. Peripheral blood Treg level is irrelevant to the status of disease.

Key words: non-Hodgkin's lymphoma, peripheral blood $CD4^+CD25^{high}$ CD127low Treg, clinical characteristic

Malignant lymphoma is a malignant tumor originated from lymph node or extranodal lymphoid tissue. Since lymphoma occurs in immune cells, tissues and organs, and AIDS patients are more prone to lymphoma, other immune suppression status, such as rheumatoid arthritis, Sjogren syndrome and organ transplant, may increase the risk for lymphoma occurrence,[1-3] the correlation between lymphoma and autoimmune suppression has thus always been of great concern, but no consensus has been reached yet.

Regulatory T cells (Treg) are a group of immune suppressive cells that have been intensively studied in recent years. Treg is closely correlated with autoimmune diseases and tumors.[4-6] With regard to lymphoma, the relationship between Treg level and the disease is not yet conclusive, and detection method for Treg has not been standardized, either. Most previous studies have labeled CD4 and CD25 on Treg, but CD25 is also expressed on the surface of functional T cells.[7] Moreover, currently no standard definitions have been set for low and high expression of CD25, the accuracy and robustness of Treg detection by CD4 and CD25 labeling are therefore compromised. Some subsequent studies suggested that CD127 expression on cell surface was negatively related to the forkhead/winged helix transcription factor p3 (FoxP3),[8] while FoxP3 has been proven the most reliable and specific marker of Treg.[9,10] Treg shows low level expression of CD127, while autoactivated T cells have high level expression of

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CD127, therefore Treg and activated T cells can be well distinguished by CD127;[11] in addition, down-regulated expression of CD127 is well correlated to the moderate-high expression of CD25, thus low level expression of CD127 is a good surrogate for FoxP3 as a marker for Treg detection.[8, 11] Combined labeling of CD4, CD25 and CD127 allows accurate detection of Treg level. Using flow cytometry with concomitant labeling of CD4, CD25 and CD127, our study aimed to detect and compare the peripheral blood $CD4^+CD25^{high}CD127^{low}$ Treg levels in NHL patients and healthy adults and analyze the correlation between peripheral blood Treg level and clinical features of the tumor.

Material and methods

Sample sources

A total of 99 treatment-naive patients with non-Hodgkin's lymphoma (NHL), who were treated in Sun Yet-sen University Cancer Center from December 2006 to March 2008, were selected. With a median age of 48 years (range, 3–82 years), 63 of them were male and 36 female. As for clinical staging, the disease was rated as stage Ⅰ in 20 patients, stage Ⅱ in 28 patients, stage Ⅲ in 25 patients and stage Ⅳ in 26 patients (Ann Arbor staging system was used for those aged >18 years, and St Jude staging system for those aged ≤ 18). With regard to other clinical features, 11 patients had bulky mass, 40 had elevated LDH level, 42 had B symptoms and 8 had spinal cord invasion (out of 90 evaluable patients). As for short-term efficacy, complete response (CR) was seen in 58 patients (out of 90 evaluable patients). Concomitant hepatitis B virus (HBV) infection was seen in 21 patients, and 29 patients were smokers and/or alcohol addicts. By pathology, B cell lymphoma was seen in 63 patients, of which 50 patients had diffuse large B cell lymphoma (DLBCL); 35 patients had T cell lymphoma; cell type was unknown in one patient. As for IPI score in patients with DLBCL, 26 patients scored 0-1, seven patients scored 2, 11 patients scored 3 and six patients scored 4–5. A total of 31 healthy adults who had normal results in physical examinations were included into normal control group. Peripheral blood sample was obtained from patient group and normal control group and was used for detection of $CD4^+CD25^{high}CD127^{low}$ Treg level, with flow cytometry and combined labeling of CD4, CD25 and CD127.

Material for the experiments

Flow cytometer FACSCalibur (Becton Dickinson; US) was used. Reagents included PE-Cy5 labeled mouse anti-human CD25 antibody, FITC labeled mouse anti-human CD4 antibody, PE labeled mouse anti-human CD127 antibody and corresponding control IgGs (by Becton Dickinson; US), and self-prepared hemolysin and PBS solution.

Detection method

Requirements for the samples Heparin-anticoagulated whole blood was taken after fasting overnight (sample was taken right before treatment in NHL patients and during routine physical examination in healthy adults). Detection was performed using fresh sample; staining was conducted with in 24 hours after sampling and detection conducted in flow cytometer within 24 hours after staining.

Detection steps An amount of 10 μL PE-Cy5 labeled mouse anti-human CD25 antibody, 10 μL FITC labeled mouse anti-human CD4 antibody and 10 μL PE labeled mouse anti-human CD127 antibody was added, then 50 μL of heparin-anticoagulated peripheral blood was added. When well blended, the sample was incubated light-shielded at room tempearture for 20 min. An amount of 2 mL hemolysin was added later, and the sample underwent light-shielded incubation for another 20 min at room temperature. Later, the sample was centrifuged at 1 000 r/min for 5 min, then supernatant was removed. After being washed with 2 mL PBS solution once, the sample underwent another centrifugation at 1 000 r/min for 5 min, and then supernatant was again removed. After being added with 0.3 mL of 1% paraformaldehyde solution, the sample underwent detection in flow cytometer FACSCalibur, and percentage of $CD4^+CD25^{high}CD127^{low}$ Treg/$CD4^+$ T cells in peripheral blood was analyzed by the software Cellquest (5 000 lymphocytes were calculated).

Statistical processing

Statistical analyses were conducted by statistical analysis software SPSS15.0. The difference in peripheral blood $CD4^+CD25^{high}CD127^{low}$ Treg level between NHL group and normal control group was analyzed with *t* test; in NHL patients, differences of peripheral blood Treg level between varied clinical grades were analyzed using analysis of variance, and differences of peripheral blood Treg level between different subgroups by qualitative clinical features analyzed using *t* test. In all these tests, two-sided $P<0.05$ indicated statistically significant difference. The correlations between peripheral blood Treg level in NHL patients and quantitative clinical features were analyzed by correlation tests, with Pearson test for normally distributed quantitative features and Spearman test for non-normally distributed quantitative features. Two-sided $P<0.05$ indicated statistical significance.

Results

Comparison of peripheral blood $CD4^+CD25^{high}CD127^{low}$ Treg level between healthy adults and treatment-naive NHL patients

Peripheral blood $CD4^+CD25^{high}CD127^{low}$ Treg level was 8.07±1.90 in healthy adults and 11.20±4.40 in treatment-naive NHL patients (Fig. 1). Statistically significant difference was seen between them ($t=5.60$; two-sided $P<0.001$; 95% CI: 2.02–4.23).

Correlation between peripheral blood $CD4^+CD25^{high}CD127^{low}$ Treg level and clinical features in NHL patients

Among NHL patients, peripheral blood $CD4^+CD25^{high}CD127^{low}$ Treg level was higher in male patients than in female patients, and higher in smoker and/or alcohol addict than in non-smoker and/or non-alcohol addict, both with statistically significant differences ($P<0.05$). Whereas no significant correlation was seen in NHL patients between peripheral blood Treg level and clinical features including age, staging, B symptoms, bulky mass,

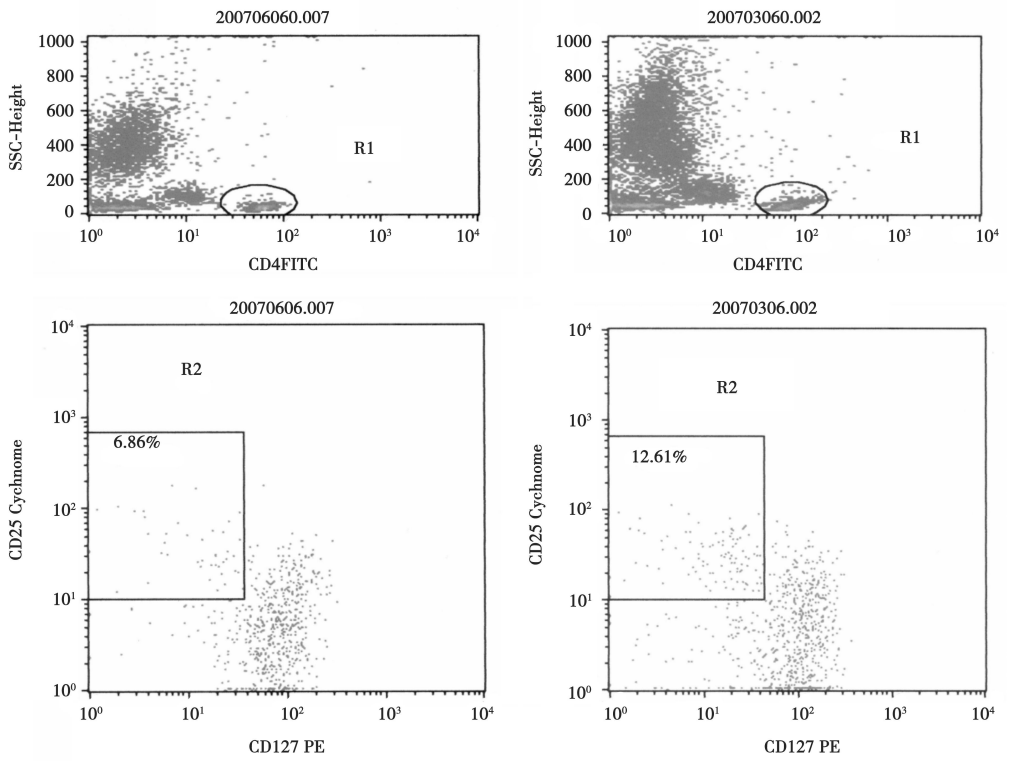

Figure 1 Peripheral blood $CD4^+CD25^{high}CD127^{low}$ Treg levels of a healthy adult and a non-Hodgkin's lymphoma (NHL) patient

Figure 1A shows the peripheral blood $CD4^+CD25^{high}CD127^{low}$ Treg level of a healthy adult; Figure 1B shows the peripheral blood $CD4^+CD25^{high}CD127^{low}$ Treg level of a NHL patient; R1 represents peripheral blood $CD4^+$ cell population; R2 represents peripheral blood $CD4^+CD25^{high}CD127^{low}$ Treg population.

LDH level, pathological subtype, short-term efficacy and HBV infection, with statistically insignificant differences between these clinical subgroups (Table 1).

A total of 50 DLBCL patients were selected for the analysis on peripheral blood $CD4^+CD25^{high}CD127^{low}$ Treg level, which revealed that peripheral blood $CD4^+CD25^{high}CD127^{low}$ Treg level

Table 1 Relationship between peripheral blood Treg level and clinical characteristics of patients with non-Hodgkin's lymphoma (NHL)

Characteristic	Item	Number	Mean	Standard deviation	*t*	*P*	95% CI
Sex	Male	63	11.92	4.44	2.197	0.030	0.19–3.77
	Female	36	9.94	4.08			
B symptom	Yes	42	11.00	4.94	−0.377	0.707	−2.12–1.44
	No	57	11.34	3.98			
Bulky disease	Yes	11	12.01	2.20	0.651	0.517	−1.88–3.72
	No	88	11.10	4.60			
Bone marrow involvement	Yes	8	10.56	3.53	−0.589	0.557	−4.19–2.27
	No	82	11.52	4.46			
HBV infection	Yes	21	11.70	4.61	0.585	0.560	−1.52–2.79
	No	78	11.06	4.36			
Pathologic subtype	B-cell	63	11.55	4.35	0.958	0.340	−0.95–2.74
	T-cell	35	10.65	4.52			
Short term response	CR	58	10.86	3.94	−0.481	0.632	−2.47–1.51
	Not CR	22	11.34	4.14			
Smoking, addict to drink, or both	Yes	29	12.57	4.32	2.031	0.045	0.04–3.84
	No	70	10.63	4.33			
Stage[a]					0.847	0.472	
Age[b]					−0.078	0.442	
LDH level[b]					0.102	0.315	

[a]ANOVA, F value; [b]correlation analysis, coefficient of correlation.

was higher in DLBCL patients with bad habits (smoking or alcohol addict) than in those without these bad habits, with statistically significant difference. No significant correlation was seen between Treg level and clinical features including age, serum LDH level, IPI score, staging, gender, B symptoms, bulky mass, spinal cord invasion, HBV infection, GCB/non-GCB and short-term efficacy, with statistically insignificant difference within these clinical subgroups (Table 2).

Table 2 Relationship between peripheral blood Treg level and the clinical characteristics in patients with diffuse large B-cell lymphoma (DLBCL)

Characteristic	Item	Number	Mean	Standard deviation	*t*	*P*	95% CI
Sex	Male	30	11.60	4.83	0.893	0.376	−1.42–3.69
	Female	20	10.47	3.65			
B symptom	Yes	18	10.62	4.65	−0.632	0.530	−3.44–1.79
	No	32	11.44	4.29			
Bulky disease	Yes	4	12.32	1.96	−0.552	0.584	−5.91–3.36
	No	46	11.05	4.54			
Bone marrow involvement	Yes	1	3.91		−1.776	0.083	−16.01–1.01
	No	46	11.41	4.18			
HBV infection	Yes	13	11.25	4.26	−0.092	0.927	−3.01–2.74
	No	37	11.11	4.49			
Pathologic subtype[a]	GCB	9	9.61	3.76	0.378	0.708	−2.65–3.85
	NonGCB	21	10.21	4.07			
Short term response	CR	32	10.09	3.80	−1.759	0.087	−5.88–0.41
	Not CR	8	12.82	4.49			
Smoking and/or addicted to drinking	Yes	15	13.56	4.30	2.701	0.010	0.88–6.01
	No	35	10.11	4.06			
Stage[b]					0.288	0.834	
IPI[b]					0.992	0.405	
Age[c]					−0.166	0.249	
LDH level[c]					−0.164	0.255	

[a]Differentiate pathologic subtype by immunohistochemistry staining of CD10, Bcl-6, and Mum-1. Only 30 cases can be evaluated.
[b]ANOVA, *F* value; [c]correlation analysis, coefficient of correlation. GCB, germinal center B cell-like; CR, complete response.

Discussions

Lymphoma is a malignant tumor originated in lymph nodes and/or extranodal lymphoid tissue. Since lymphoma occurs in immune cells, tissues and organs, and patients with immune defects tend to have increased risk for lymphoma occurrence,[1,2] lymphoma occurrence is now considered related to autoimmune status. In 1975, Japanese scientists found that suppressor T cells could interfere with valid anti-tumor immunity.[12] In 1995, another Japanese scientist found that $CD4^+CD25^+$ T cells showed immune suppression, and had a critical role in preventing autoimmune diseases.[7] Numerous studies on Treg have focused on autoimmune diseases, tumors, transplant and pregnancy. With respect to tumors, Treg has important role in inhibiting the anti-tumor immune response in the body by suppressing the activity of cytotoxic T cells and preventing apoptosis in tumor cells.[6,13] Meanwhile, tumor cells per se can induce aggregation and proliferation of Treg in local tissue and stop dendritic cells from maturing and presenting tumor antigens to T cells.[14] Lymphoma induces local and systemic Treg production.[15]

In lymphoma, the relationship between Treg and lymphoma is still controversial. Both elevated and decreased Treg levels were observed; some studies indicated positive correlation with prognosis, but opposite opinion was also suggested; different conclusions were reached for different subtypes and for peripheral blood and local tumor tissue as well.[15-21]

Before 2007, detection methods for Treg mostly involved CD4 and CD25 labeling on Treg. Since CD25 is also expressed on activated effector T cells besides on Treg,[7] and there is no standard definition for high level expression of CD25, combined labeling of these two markers is now considered an inaccurate method in detecting peripheral blood Treg level. Therefore, the results from studies that detected Treg with this labeling method are much less valuable. Ever since July 2006 when scientists found that lowly-expressed extracellular CD127 could be a replacement for FoxP3 level as a simple and accurate marker of Treg, more and more studies at home and abroad have been using combined labeling of CD4, CD25 and CD127 in detecting peripheral blood Treg level in both normal individuals and tumor patients.[22-24] In 2008, foreign scientists reported a study using combined labeling of CD4, CD25, CD127 and FoxP3 in detecting Treg levels in peripheral blood and tumor tissue of NHL patients, and suggested that Treg level was remarkably higher in NHL patients than in healthy individuals and was significantly related to tumor burden and staging, rather than pathological classification.[15] But these study results were not representative due to a small subject number, which included 30 treatment-naive NHL patients and 13 healthy individuals.

In our study, we used combined labeling of CD4, CD25 and CD127, which was currently considered a substantially accurate detection method, to measure the peripheral blood Treg level in

99 treatment-naive NHL patients and 31 healthy adults. The results indicated that treatment-naive NHL patients had elevated peripheral blood Treg level as compared to healthy adults in control group, with statistically significant difference. These results were in consistence with those reported by foreign and domestic scientists.[15, 25] It was therefore indicated that immune suppression did exist in patients with lymphoma. Treg might have certain roles in the occurrence of lymphoma, that is, elevated Treg level had suppressed the immune system of the body; since anti-tumor immunity was inhibited, the body could not get rid of the tumor cells effectively and thus lymphoma developed; meanwhile, lymphoma cells, in turn, induced production and local aggregation of Treg[15] and thereby further aggravated the immune suppression, starting a vicious circle.

Our study also found that patients with bad habits (smoking and/or alcohol addiction) had higher peripheral blood Treg level than those without these habits, with statistically significant difference. It is well-known that smoking is a risk factor for various solid tumors, such as lung cancer, oropharyngeal cancer, esophageal cancer, gastric cancer, bladder cancer, hepatic cancer and renal cancer. In 1998, a foreign study reported that smoking increased the risk for NHL occurrence.[26] Recently, a European prospective study suggested that the incidence of NHL in smokers more than tripled as compared with that in non-smokers,[27] indicating possible correlation between smoking and lymphoma occurrence. Is it because smoking inhibits systemic immunity or smoking results in mutation in somatocytes and thus leads to lymphoma? Are both these mechanisms involved? These questions will have to be answered by further studies. Alcohol addiction was also reported to increase the risk for NHL occurrence,[26] but the mechanism had yet to be revealed by further investigations. In our study, male patients had significantly higher peripheral blood Treg level than female patients. Bad habits were considered confounding factor in such difference, since all the bad habits were seen in male patients but not in female patients. Therefore, the relationship between gender and peripheral blood Treg level needed to be further confirmed.

In our study, analyses on the relationship between the peripheral blood Treg level in lymphoma patients and their clinical features suggested that peripheral blood Treg level was not significantly related to clinical features, including age, staging, IPI score, B symptoms, bulky mass, LDH level, spinal cord invasion, pathological subtypes, short-term efficacy and HBV infection; Treg level was not related to tumor burden or disease staging, either; similar results were seen for the analysis within the same subtype of lymphoma (diffuse large B cell lymphoma). These indicated that immune suppression had certain role in the occurrence of lymphoma, but might not be related to subsequent development once the disease was established. A foreign study also suggested that Treg had a major role in the immune escape at early stage of B cell lymphoma occurrence, but not in later development of the disease.[28] This study used lymphoma cell line A20B to establish mouse models; in a group of mice, Treg was removed by anti-CD25 antibody (PC16) before tumor implant, and the results suggested that tumor-free survival rate was 70% at 50 days after tumor implant, while in control group, the corresponding survival rate was 0; in the mice where Treg was removed by anti-CD25 antibody after tumor implant, no tumor shrinking effect was observed.[28] Hence, we believed the immune suppression induced by Treg might be related to the occurrence of lymphoma, but not to the later development once the disease was established.

Since Treg level was elevated in patients with lymphoma, the occurrence of lymphoma might be related to Treg, indicating that tumor might be treated and prevented by targeting Treg. Numerous foreign studies have tried to treat tumors by targeting Treg, mostly using anti-CD25 monoclonal antibody[29] and ONTAK (a complex of IL-2 receptor binding domain and diphtheria toxin, also known as denileukin-2).[30] However, these agents are not widely used so far. The main reason for this is that such immunotherapy is not specific. Since activated CD4 and CD8 cytotoxic T cells also express CD25, clearance of cells expressing CD25 may do more harm than good; in addition, extensive clearance of Treg may increase the susceptibility to autoimmune diseases.[31] Now that we proved lowly-expressed extracellular CD127 in combined with CD4 and CD25 an accurate method to label Treg, whether or not we can develop targeting agents, which modulate both CD25 and CD127, to treat or even prevent tumors including lymphoma by better regulating Treg remains to be seen in further investigation.

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