

# Immunophenotype and increased presence of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells in patients with acute lymphoblastic leukemia

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Received August 11, 2011; Accepted November 7, 2011

DOI: 10.3892/ol.2011.499

**Abstract.** Acute lymphoblastic leukemia (ALL), cancer of the white blood cells, is a heterogeneous disease that mainly occurs due to the malignant cloning of original and naive lymphocytes. The aim of this study was to explore the immunophenotype, the percentage of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (Tregs) and the expression of cytokines interleukin (IL)-2, IL-10 and TGF- $\beta$  in patients with ALL. The immunophenotype and levels of CD4<sup>+</sup>CD25<sup>+</sup> Tregs were detected using flow cytometry in the peripheral blood of 35 ALL patients, with 18 healthy individuals being selected as controls. The results suggested that 22 patients had B cell ALL (B-ALL) and 13 had T cell ALL (T-ALL) among the 35 ALL patients. In B-ALL patients, the surface antigen CD19 was most commonly expressed; in T-ALL patients, CD7 was most common. Furthermore, the percentage of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells in the peripheral blood of B-ALL and T-ALL patients was higher compared to that of healthy individuals ( $P < 0.05$ ). Additionally, IL-10 and TGF- $\beta$  levels in cell culture supernatants from B-ALL and T-ALL patients were higher compared to those in the controls ( $P < 0.05$ ); IL-2 levels were lower in ALL patients. No significant differences were observed in the levels of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells, IL-2, IL-10 or TGF- $\beta$  in B-ALL versus T-ALL patients. The authors concluded that CD19 and CD7 may serve as diagnostic markers of B-ALL and T-ALL, respectively. The increased presence of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells and the altered levels of secreted cytokines are indicative of an immunosuppressive mechanism in the pathogenesis of ALL.

## Introduction

Acute lymphoblastic leukemia (ALL), cancer of the white blood cells, is a heterogeneous disease, which occurs mainly due to the malignant cloning of original and naive lymphocytes. ALL

is characterized by massive proliferation, extensive infiltration and inhibition of normal hematopoiesis. Leukemic cells are identified based on their morphology, immunophenotype, cytogenetics and molecular abnormalities, although the cause of ALL remains unclear (1).

Lymphocyte typing reveals two subtypes of ALL; B cell (B-ALL) and T cell (T-ALL) (2). Advances in cellular immunology have made immunophenotype analysis by flow cytometry an essential tool in the diagnosis and classification of ALL, in that it is now far easier to distinguish the source and differentiation stages of ALL accurately, providing a reference point for clinical treatment (3).

CD4<sup>+</sup>CD25<sup>+</sup> T regulatory cells (Tregs) are a recently discovered type of immune suppression regulatory cell, first reported in 1995 by Sakaguchi *et al* (4). CD4<sup>+</sup>CD25<sup>+</sup> Treg cells have low reactive and immunosuppressive functions. The unique immune response of these cells suggests that, in ALL pathogenesis, CD4<sup>+</sup>CD25<sup>+</sup> Treg cells has certain negative effects on the body's immune response. To gain insight into this potential mechanism of ALL pathogenesis, we compared the percentage of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells in peripheral blood from ALL patients and healthy controls. We also determined the levels of cytokines IL-2, IL-10 and TGF- $\beta$ , associated with immune function, secreted by these cells.

## Subjects and methods

**Study subjects.** Thirty-five newly diagnosed ALL patients were selected at the Affiliated Hospital of Hainan Medical College, China, between January 2008 and January 2010. Of the 35 patients, 19 were male and 16 were female. The age range of the patients was 16-56 years (median age 31). Diagnoses were confirmed with bone marrow smears, cell histochemical staining and clinical manifestation, and classified according to the FAB diagnostic criteria. For the control population, 18 healthy individuals from the health examination center of our hospital were sampled during the same period, comprising 10 males and 8 females, aged 22 to 50 years (median 30).

## Research methods

**Immunophenotype analysis.** Venous blood (5 ml) was collected from each individual, and ALL samples were obtained before chemotherapy began. Within 2 h, samples were directly labeled with three-color fluorescence using a monoclonal antibody. Briefly, 20  $\mu$ l monoclonal antibody was

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**Key words:** acute lymphoblastic leukemia, immunophenotype, CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cell, interleukin-2, interleukin-10, transforming growth factor- $\beta$

Table I. Expression of differential antigens in 35 patients with acute lymphoblastic leukemia [n (%)].

Types	n	CD2	CD3	CD7	CD19	CD20	CD22
B-ALL	22	0	1 (4.5)	0	22 (100)	9 (40.9)	14 (63.6)
T-ALL	13	9 (69.2)	10 (76.9)	13 (100)	0	0	0
Total	35	9 (25.7)	11 (31.4)	13 (37.1)	22 (62.9)	9 (25.7)	14 (40.0)

Types	n	CD13	CD14	CD33	CD117	HLA-DR	CD34
B-ALL	22	5 (22.7)	0	2 (9.1)	0	21 (95.5)	19 (86.4)
T-ALL	13	0	1 (7.7)	2 (15.4)	3 (23.1)	7 (53.8)	6 (46.2)
Total	35	5 (14.3)	1 (2.9)	4 (11.4)	3 (8.6)	28 (80.0)	25 (71.4)

B-ALL, B cell acute lymphoblastic leukemia; T-ALL, T cell acute lymphoblastic leukemia.

added to 100  $\mu$ l sample. The solutions were mixed evenly and stored in the dark at room temperature for 5 min, and then for another 10 min following hemolysis. Subsequently, samples were centrifuged for 5 min, supernatants were discarded and sediment was washed twice with phosphate-buffered saline (PBS) prior to detection. The monoclonal antibodies used were: for the T series, CD2, CD3 and CD7; for the B series, CD19, CD20 and CD22; for myeloid, CD13, CD14, CD33, CD117 and CD41a; and non-series-related, HLA-DR, CD34 and CD45. Antibody combinations used were: i) IgG1-FITC/IgG1-PE/CD45-PerCP, ii) CD14-FITC/CD13-PE/CD45-PerCP, iii) CD33-FITC/CD117-PE/CD45-PerCP, iv) CD3-FITC/CD2-PE/CD45-PerCP, v) CD7-FITC/HLA-DR-PE/CD45-PerCP, vi) CD19-FITC/CD34-PE/CD45-PerCP, vii) CD20-FITC/CD22-PE/CD45-PerCP, and viii) CD41a-FITC/Pgp-PE/CD45-PerCP. Three fluorophores were used [fluorescein isothiocyanate (FITC), phycoerythrin (PE) and peridinium chlorophyll protein (PerCP)], all purchased from Becton-Dickinson (Franklin Lakes, New Jersey, USA). A FACScan flow cytometer (Becton-Dickinson) and a 488 nm excitation light source were used. Standard fluorescent microspheres and CellQuest software were used prior to determination, and 10,000 cells were analyzed per tube. Abnormal cells were analyzed by CD45/SSC gating. Samples were considered positive for CD34 if a minimum of 10% of cells was detected with this antibody; for other proteins, samples were considered positive if a minimum of 20% of cells was detected with the respective antibodies.

**CD4<sup>+</sup>CD25<sup>+</sup> Treg cell detection.** Peripheral blood (100  $\mu$ l) was collected, placed in flow test tubes and added into negative $\gamma$ 1/ $\gamma$ 2 $\alpha$  control tubes of the same type. Tubes were incubated at 4°C for 30 min, and 3,000  $\mu$ l hemolysin was added to each tube. Samples were mixed and incubated at room temperature for 12 min, followed by centrifugation at 1,000 rpm for 5 min. Supernatants were discarded, and 500  $\mu$ l sheath liquid was added. Flow cytometry was used for detection, and CellQuest software was used to analyze the data. The percentage of positive cells was recorded, and the non-specific control value was subtracted.

**CD4<sup>+</sup>CD25<sup>+</sup> Treg cell isolation.** Venous blood (5 ml) was diluted with an equivalent volume of normal saline. Diluted

white blood cell suspension was slowly added at a 1:1 ratio to the surface of lymphocyte separation medium, then centrifuged at 2,000 rpm for 20 min. A single layer of nucleated cells was withdrawn and washed twice with PBS. A quantity of the freshly-isolated peripheral blood mononuclear cells (PBMC) were removed for CD4<sup>+</sup>CD25<sup>+</sup> Treg cell isolation on a magnetic bead separation kit (Miltenyi Biotechnology Co., Ltd., Germany). Flow cytometry was used to analyze cell purity. One milliliter CD4<sup>+</sup>CD25<sup>+</sup> Treg cells (1x10<sup>6</sup>/ml) was added to each well of a 24-well culture plate, with replicates. The plate was then incubated in a 37°C 5% CO<sub>2</sub> incubator.

**IL-2, IL-10 and TGF- $\beta$  detection.** CD4<sup>+</sup>CD25<sup>+</sup> Treg cells were cultured for 48 h. Following centrifugation at 1,000 rpm for 10 min, cell culture supernatants were collected, and double-antibody sandwich ELISA (Shanghai Senxiong Technology Co., Ltd., China) was used to detect IL-2, IL-10 and TGF- $\beta$  secretions in the supernatant, according to the manufacturer's instructions.

**Statistical analysis.** SPSS 11.5 statistical software was used for the statistical analysis. Test results are expressed as the means $\pm$ standard deviation. IL-2, IL-10 and TGF- $\beta$  levels were compared by single-factor analysis of variance (one-way ANOVA) and multiple comparisons (LSD) were based on ANOVA. The analysis used a two-sided test, with an  $\alpha$  level of 0.05, and P<0.05 considered statistically significant.

## Results

**Antigen expression in ALL.** Of the 35 ALL patients, 22 (62.9%) had B-ALL and 13 (37.1%) had T-ALL (Table I). Seven (31.8%) B-ALL patients exhibited myeloid antigen expression, whereas this expression was evident in 6 (46.2%) T-ALL patients. The B-ALL patients exhibited CD19 expression, while fewer patients tested positive for HLA-DR (95.5%), CD34 (86.4%) and CD22 (63.6%). Of the 7 B-ALL patients with myeloid antigen expression, 5 patients were also positive for CD13 and 2 patients were positive for CD33. In contrast, the T-ALL patients tested positive for CD7 expression; the expression of CD3, CD22 and HLA-DR was evident in fewer patients (76.9, 69.2 and 53.8%, respectively). Of the 6 T-ALL

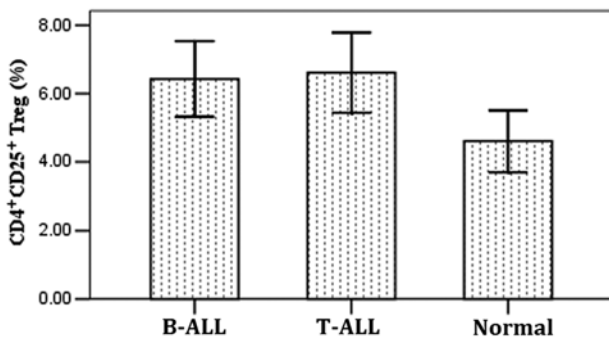


Figure 1. Percentage of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells in ALL patients and healthy individuals. B-ALL, B cell acute lymphoblastic leukemia; T-ALL, T cell acute lymphoblastic leukemia.

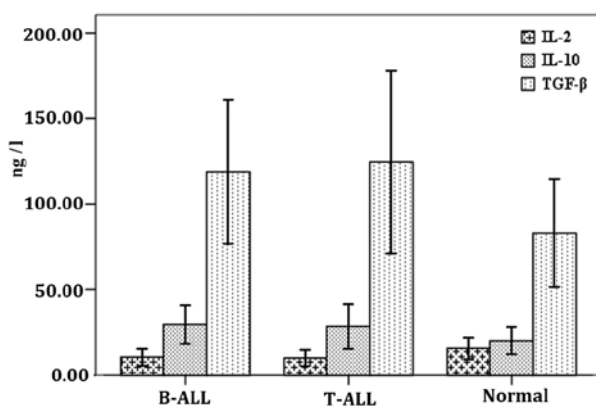


Figure 2. Levels of IL-2, IL-10, and TGF-β secreted by cultured CD4<sup>+</sup>CD25<sup>+</sup> Treg cells from ALL patients and healthy individuals. B-ALL, B cell acute lymphoblastic leukemia; T-ALL, T cell acute lymphoblastic leukemia.

patients with myeloid antigen expression, 3 patients were also positive for CD117 expression, 2 patients for CD33 expression and 1 patient for CD14 expression. The results showed that CD19 serves as the most effective marker for B-ALL, and CD7 is the most effective marker for T-ALL.

**Percentage of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells.** Using magnetic bead isolation, CD4<sup>+</sup>CD25<sup>+</sup> Treg cells were isolated from the peripheral blood of ALL patients and healthy individuals. The mean percentages of these cells were  $6.42 \pm 1.11\%$  in B-ALL patients,  $6.61 \pm 1.18\%$  in T-ALL patients, and  $4.61 \pm 0.91\%$  in the healthy controls (Fig. 1). The difference in percentages of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells between groups was statistically significant ( $F=18.927$ ,  $P<0.01$ ). The percentages of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells were significantly higher in B-ALL and T-ALL patients compared to the healthy individuals ( $P<0.05$ ), but no significant difference was found in the percentages between B-ALL and T-ALL patients. Thus, CD4<sup>+</sup>CD25<sup>+</sup> Treg cells appear to be involved in the pathogenesis of ALL. We used flow cytometry to verify the purity of isolated CD4<sup>+</sup>CD25<sup>+</sup> Treg cells. Cell purity was  $>90\%$  (data not shown), making this population suitable for further experimentation.

**IL-2, IL-10 and TGF-β levels.** The increase in CD4<sup>+</sup>CD25<sup>+</sup> Treg cells in patients with ALL indicates the suppression of

the immune system. To confirm the apparent immunosuppressive action in ALL, we investigated the secretion of several cytokines involved in immune function, such as IL-2, IL10 and TGF-β, in the supernatant of cultured CD4<sup>+</sup>CD25<sup>+</sup> Treg cells (Fig. 2). The mean levels of IL-2 secreted in Treg cell culture supernatants of B-ALL patients, T-ALL patients and healthy controls were  $10.36 \pm 4.82$  ng/l,  $9.85 \pm 4.72$  ng/l and  $15.49 \pm 6.33$  ng/l, respectively. The levels of IL-2 in patients with either type of ALL were significantly lower compared to the healthy controls ( $P<0.05$ ). In contrast, secreted IL-10 levels were significantly higher in CD4<sup>+</sup>CD25<sup>+</sup> Treg cells from patients with ALL (B-ALL,  $29.50 \pm 11.26$  ng/l; T-ALL,  $28.37 \pm 13.08$  ng/l) compared to the healthy individuals ( $20.01 \pm 8.08$  ng/l;  $P<0.05$ ). The same was true for secreted TGF-β levels (B-ALL,  $118.90 \pm 42.23$  ng/l; T-ALL,  $124.57 \pm 53.53$  ng/l and controls,  $82.99 \pm 31.50$  ng/l;  $P<0.05$ ). No significant differences were observed in IL-2, IL-10 or TGF-β levels between B-ALL and T-ALL patients.

## Discussion

Recent efforts have generated new data on the differentiation and development of lymphocytes. In particular, the generation of lymphocyte surface antigen monoclonal antibodies has uncovered a significant amount of information regarding the development and biological characteristics of lymphocytes. Monoclonal antibodies are capable of detecting the different stages of lymphocyte development and maturation, and thus are used in immunophenotyping to identify the unique antigen combinations expressed during the differentiation stages of normal cells (5).

The immunophenotyping of acute lymphoblastic leukemia by flow cytometry, based on the detection of surface and internal differentiation antigens, determines their cell type (B or T series) and differentiation stages to aid in diagnosis (6). The application of monoclonal antibodies provides more accurate immunophenotyping, improving the differential diagnosis of leukemia and lymphoma, as well as determining the source of malignant cells and their degree of differentiation. Of note, this typing method is more accurate than the FAB L1, L2, L3 method, thus permitting more individualized treatment regimens (7). In the present study, we have shown that the surface marker, CD19, is most common in B-ALL patients, whereas, CD7, is most common in T-ALL patients. This information is valuable in determining the immunophenotypes of ALL and should enable better diagnosis and evaluation of prognosis, particularly when combined with flow cytometry.

Treg cells are a recently discovered type of cell with low reactivity and immune suppression functions, which confer a natural antigen-specific tolerance (8). Treg cells play significant roles in immune homeostasis, autoimmune diseases, allergic diseases and transplantation tolerance, among other functions. Notably, T cells suppress anti-tumor immunity; thus, the occurrence and progression of certain tumors may be correlated to Treg function (9,10). Numerous studies have described the correlation between solid tumors and Treg cells: lymphocytes infiltrating the tumor cells include CD4<sup>+</sup> and CD8<sup>+</sup> T cells, as well as a large number of Treg cells, mainly CD4<sup>+</sup>CD25<sup>+</sup>. In patients with breast, ovarian, lung, pancreatic, colorectal and liver cancers, malignant mela-

noma, and other cancer types, the proportion of Treg cells is high in peripheral blood and local tissues, and the number of Treg cells is negatively correlated with the extent of tumor progression and prognosis. Furthermore, the removal of Treg or the suppression of its inhibitory function restores anti-tumor immunity (11).

In hematologic malignancies, Marshall *et al* first reported increased numbers of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells infiltrating lymphocytes and mononuclear cells in the peripheral blood of Hodgkin's lymphoma patients. This infiltration inhibits T-cell function through the secretion of cytokines and inter-cell contact (12). Results of the present study showed that the percentage of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells was significantly higher in B-ALL and T-ALL patients than in healthy individuals. This increase in ALL patients may be associated with tumor occurrence. However, we also revealed that the secretion of cytokines IL-2, IL-10 and TGF- $\beta$  was altered in ALL patients. IL-2 is an essential immune regulatory factor produced by helper T lymphocytes, capable of promoting T-cell proliferation, the secretion of antibodies by B cells and T-cell killing ability, as well as inducing LAK cells and enhancing the activity of NK cells (13). We observed a reduced secretion of IL-2 in ALL patients. In contrast, IL-10 is a strong immunosuppressive factor with multi-directional biological activity, capable of changing the body's immune response and major histocompatibility complex II (MHC II) antigen expression, and mediating inter-regulation between the Th1 and Th2 cells (14). TGF- $\beta$  is also an immunosuppressive factor, capable of inhibiting the proliferation of active immune cells, the differentiation of lymphocytes and the production of interferon- $\gamma$  and tumor necrosis factor- $\alpha$  in PBMC; this cytokine has significant regulatory effects on the immune functions of cells (15). We detected an increased secretion of IL-10 and TGF- $\beta$  from cultured CD4<sup>+</sup>CD25<sup>+</sup> Treg cells compared to those from healthy individuals. These findings indicate that CD4<sup>+</sup>CD25<sup>+</sup> Treg cells may suppress immune function in ALL through the downregulation of IL-2 and the upregulation of IL-10 and TGF- $\beta$ .

In conclusion, CD4<sup>+</sup>CD25<sup>+</sup> Treg cells play a significant role in tumor cells escaping immunity, leading to the immune tolerance of tumors. Further studies are required to investigate the inhibition mechanism of tumor immunity by these Treg cells to provide a new approach for tumor immunotherapy. Further studies are also required to confirm the approach by which CD4<sup>+</sup>CD25<sup>+</sup> Treg cells regulate cytokine secretion using the body's own cells.

## References

1. Han X and Bueso-Ramos CE: Advances in the pathological diagnosis and biology of acute lymphoblastic leukemia. *Ann Diagn Pathol* 9: 239-257, 2005.
2. Onciu M: Acute lymphoblastic leukemia. *Hematol Oncol Clin North Am* 23: 655-674, 2009.
3. Qiu Y, Wang G, Liu YJ, and Shan LJ: Analysis of immunophenotypes of acute lymphoblastic leukemia by three color flow cytometry. *Zhongguo Shi Yan Xue Ye Xue Za Zhi* 17: 442-444, 2009.
4. Sakaguchi S, Sakaguchi N, Asano M, Itoh M and Toda M: Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor  $\alpha$ -chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 155: 1151-1164, 1995.
5. Krawczyńska A and Robak T: Monoclonal antibodies in diagnosis of acute leukemias. *Przegl Lek* 53: 20-25, 1996.
6. Nachman J: Clinical characteristics, biologic features and outcome for young adult patients with acute lymphoblastic leukaemia. *Br J Haematol* 130: 166-173, 2005.
7. Ou Y and Zhu P: Mechanism of CD4<sup>+</sup>CD25<sup>+</sup> and CD8<sup>+</sup> regulatory T cells – review. *Zhongguo Shi Yan Xue Ye Xue Za Zhi* 13: 1137-1140, 2005.
8. Gupta S: Immune homeostasis: regulatory T cells (Treg) and molecules. *J Clin Immunol* 28: 617-618, 2008.
9. Gershon RK and Kondo K: Infectious immunological tolerance. *Immunology* 21: 903-914, 1971.
10. Berendt MJ and North RJ: T-cell-mediated suppression of anti-tumor immunity: an explanation for progressive growth of an immunogenic tumor. *J Exp Med* 151: 69-80, 1980.
11. Zhang L, Dermawan KT, Jin ML, Xiong SD and Chu YW: Does chemotherapy augment anti-tumor immunotherapy by preferential impairment of regulatory T cells? *Med Hypotheses* 71: 802-804, 2008.
12. Marshall NA, Christie LE, Munro LR, Culligan DJ, Johnston PW, Barker RN, and Vickers MA: Immunosuppressive regulatory T cells are abundant in the reactive lymphocytes of Hodgkin lymphoma. *Blood* 103: 1755-1762, 2004.
13. Turka LA and Walsh PT: IL-2 signaling and CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> regulatory T cells. *Front Biosci* 13: 1440-1446, 2008.
14. Sabat R, Grütz G, Warszawska K, Kirsch S, Witte E, Wolk K and Geginat J: Biology of interleukin-10. *Cytokine Growth Factor Rev* 21: 331-344, 2010.
15. Meulmeester E and Ten Dijke P: The dynamic roles of TGF- $\beta$  in cancer. *J Pathol* 223: 205-218, 2011.