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*Original Article*

# Functional role of regulatory T cells in B cell lymphoma and related mechanisms

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**Abstract:** B cell lymphoma (BCL) has a higher degree of malignancy and complicated pathogenic mechanism. Regulatory T cells (Treg cells) are known to exert certain immune suppression functions, in addition to immune mediating effects. Recent studies have revealed the role of Treg cells in pathogenesis and progression of multiple malignant tumors. This study therefore investigated the functional role and related mechanism of Treg cells in BCL. A cohort of thirty patients who were diagnosed with BCL in our hospital between January 2013 and December 2014. Another thirty healthy individuals were recruited. Peripheral blood mononuclear cells (PBMCs) were separated and analyzed for the ratio of CD4+/CD25+ Treg cells. The mRNA expression levels of Foxp3, transforming growth factor (TGF)-β1 and interleukin (IL)-10 genes were quantified by real-time PCR, while their serum levels were determined by enzyme-linked immunosorbent assay (ELISA). Meanwhile all laboratory indexes for patients were monitored during the complete remission (CR) stage. BCL patients significantly elevated ratio of CD4+/CD25+ Treg cells, which were decreased at CR stage. mRNA levels of Foxp3, TGF-β1 and IL-10, in addition to protein levels of TGF-β1 and IL-10 were potentiated in lymphoma patients but decreased in CR patients ($P<0.05$ in all cases). CD4+/CD25+ Treg cells exert immune suppressing functions in BCL via regulating cytokines, thereby facilitating the pathogenesis and progression of lymphoma.

**Keywords:** B cell lymphoma, regulatory T cells, transforming growth factor-β1 interleukin-10, Foxp3 gene

## Introduction

Malignant lymphoma, with its increasing incidence and high mortality rate, stands as one of the most common malignant tumors in China and worldwide [1, 2]. Based on the pathological features, lymphoma has been divided as T cell lymphoma, B cell lymphoma (BCL), Hodgkin's lymphoma and NK cell lymphoma [3]. BCL has various sub-types including typical Hodgkin's lymphoma and five non-Hodgkin's lymphoma (follicular lymphoma, diffuse large B cell lymphoma, small lymphocytic lymphoma, mucosa-associated lymphoid tissue lymphoma and mantle cell lymphoma), which accounts for about 75% of non-Hodgkin's lymphoma [4-6]. BCL had a complex pathogenic mechanism, which involve multiple factors including cytokines. It has been well known that immune dysfunction had an indispensable role in BCL pathogenesis, but leaving the detailed mechanism unclear yet [7, 8].

Regulator T cell (Treg cell) is firstly discovered in autoimmune disease research, and has been found to play an important role in maintaining the homeostasis of body immune system via its dual functions of both immune suppression and immune mediation [9, 10]. The major cell phenotypes of Treg cells are CD4+, CD25+ and Foxp3+, among which the abnormal expression may lead to the abortion of immune response and consequent immune damage [11]. The decreased number or dysfunction of Treg cells has been shown to cause auto-immune diseases [12]. Recent studies also showed the participation of Treg cells in the immune escape and oncogenesis, as supported by the altered number and functions of Treg cells in tumors, suggesting their potency in mediating tumor immune response [13, 14]. The function and related mechanism of Treg cells in BCL, however, remained unclear at current stage. This study therefore investigated the possible role of

**Table 1.** Primer sequences in RT-PCR

| Genes | Forward primer (5'->3') | Reverse primer (5'->3') |
|---|---|---|
| GADPH | AGTGC CAGCC TCGTC TCATA G | CGTTG AACTT GCCGT GGGTA G |
| Foxp3 | CTCAG CGGAT CTAAA CGGAA T | CACAT TCTGG CGCTC CGTA |
| TGF-β1 | TCAGA AACTG CTCGG TCAGA | GCCTC AGGGG ATTAA AGCTC |
| IL-10 | GATCC AGGAT GAGGG GATTT | CAGGG TGTCT CCTGG TCTGT |

such cells in the pathogenesis and progression of BCL, as well as related mechanisms.

## Materials and methods

### *Patient information*

Thirty BCL patients who were admitted and diagnosed in our hospital between January 2013 and December 2014 were recruited in this study as the disease group. There were 17 males and 13 females, aging between 27 and 68 years old (average = 38±12 years). Meanwhile another thirty healthy volunteers, including 15 males and 15 females (average age = 36±17 years) were recruited as the control group. No statistically significant difference has been found between those two groups regarding general information such as sex and age ($P>0.05$). The diagnosis and typing of BCL followed the pathological classification standard stipulated by WHO. There were 8 Hodgkin's lymphoma patients, 5 case of follicular lymphoma, 12 patients with diffuse large B cell lymphoma, 2 cases of small lymphocytic lymphoma, only 1 mucosa-associated lymphoid tissue lymphoma case and 2 cases of mantle cell lymphoma. This study has been approved by the ethical committee in our hospital and has obtained written consents from patients/volunteers involved.

### *Inclusive criteria*

(1) Fitted with clinical symptoms and body signs of BCL, in addition to the confirmed diagnosis by pathological examination; (2) not received any relevant treatments including surgery, radio-/chemo-therapy or other medications; (3) at complete remission (CR) stage after the whole treatment schedule, with the disappearance of all symptoms or body signs for at least four weeks.

### *Exclusive criteria*

(1) have received treatments for BCL; (2) recurrent BCL patients; (3) complicated with other malignant tumors, diabetes, severe liver/renal dysfunctions, cardiovascular disease, systemic immune disorders or other cancer complications.

### *Sample collection and peripheral blood mononuclear cells (PBMCs) separation*

Fasted venous blood samples (10 mL) were collected from patients within 24 hours after being admitted and reaching clinical CR stage. Healthy individuals in the control group also had blood samples collected. Half of samples (5 mL) were separated for PBMCs. Remaining blood samples were centrifuged at 3,000 rpm for 15 min to collect the serum, which was then transferred into new tubes and was kept at -80°C for further enzyme-linked immunosorbent assay (ELISA).

PBMCs were separated using commercially purchases separation buffer (Haoyang Biotech, China) following the manual instruction. In brief, equal volumes of heparin-treated venous blood plus Hank's solution (Invitrogen, US) were carefully stacked on lymphocyte separation buffer. The whole mixture was then horizontally centrifuged for 2,000 rpm for 20 min. Micro capillary tubes were used to draw the white phase in the middle of the tube. The fraction was then added with five-volumes of Hank's solution, and was centrifuged at 1,500 rpm for 15 min. After gentle washing for 2 times, cells were re-suspended in RPMI1640 medium containing 10% fetal bovine serum (FBS) (Invitrogen, US). Cell number was counted by trypan blue staining.

### *Flow cytometry*

After the centrifugation, PBMCs were re-suspended in 1.5 mL flow washing buffer. After another centrifugation at 1,000 rpm for 5 min, non-specific serum was added for blocking Fc fragment receptor. After another round of washing and re-suspension, PBMCs were added with surface markers including CD4-FITC and CD25-allophycocyanin (APC) (Miltenyi Biotech, Germany). After 4°C incubation for 30 min, cells were re-suspended, washed, fixed and incubated at room temperature for 20 min. Membrane lysis buffer (Miltenyi Biotech, Germany) was then added, followed by three times of washing. Cell fractions were finally mixed with flow washing buffer for the on-line quantification.

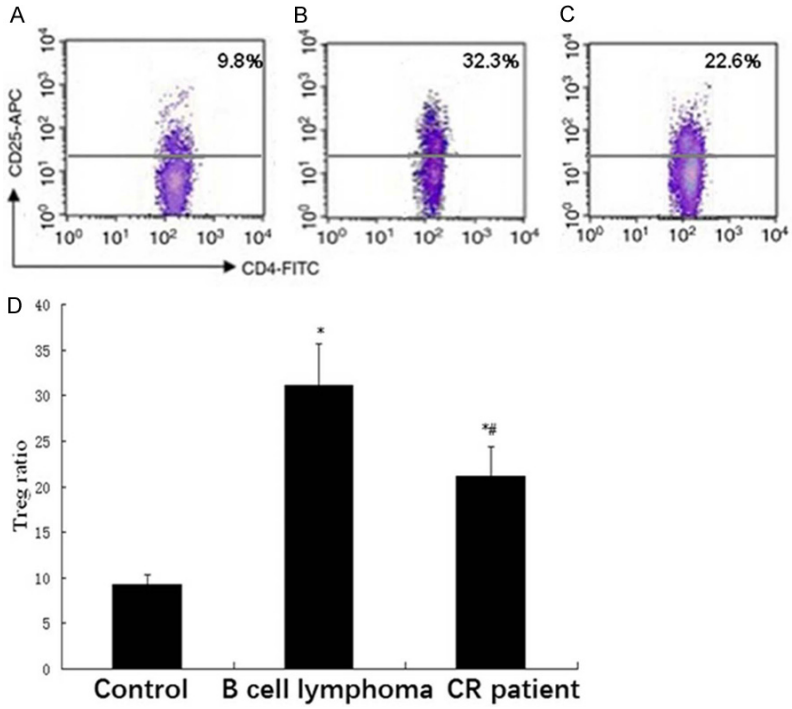


**Figure 1.** Treg cell ratio in B cell lymphoma. A. One healthy individual; B. One representative patient with B cell lymphoma at active stage; C. CR stage lymphoma patient. D. Quantitative analysis of averaged Treg cell ratio from three groups. *, $P<0.05$ compared to control group; #, $P<0.05$ compared to lymphoma group.

*ELISA*

Serum levels of transforming growth factor (TGF)-β1 and interleukin (IL)-10 were quantified by ELISA kits (BD, US) following manual instruction. In brief, serial diluted standard samples were loaded onto 96-well plate, which also contains diluted samples. After washing and addition of enzyme-linked reagents, the plate was incubated at 37°C for 30 min. Chromogenic reagent A and B were then added for 10-min incubation, which was stopped by stopping buffer. Optical density (OD) values of each well at 450 nm were measured by a microplate reader. The standard curve was firstly plotted using standard samples and was then used to deduce the sample concentration.

*RT-PCR*

Blood samples were centrifuged at 4,000 g for 10 min. After discarding the supernatants, cell pellets were lysed in Trizol (Invitrogen, US) followed by centrifugation. The supernatant was transferred to a new tube for extraction of mRNA using Trizol reagent. RT-PCR was performed using specific primers (**Table 1**) and reverse transcription kit (Invitrogen, US) following manual instruction. The reaction parameters were: 90°C denature for 30 sec, 52°C annealing for 1 min and 72°C elongation for 35 sec, repeated for 35 cycles. A fluorescent PCR cycler was used to collect the expression level. Data were analyzed using $2^{-\Delta Ct}$ method using GAPDH gene as the internal reference.

*Statistical analysis*

SPSS ver. 20.0 software package was used to process all collected data. Enumeration data were analyzed by chi-square test while measurement data were presented as mean ± standard deviation (SD) and compared by one-way analysis of variance (ANOVA). A statistical significance was defined when $P<0.05$.

## Results

*Treg cell ratios in BCL*

PBMCs were separated from BCL patients at active stage, at CR stage and from healthy controlled individuals, and were analyzed using flow cytometry. Results (**Figure 1**) showed significantly elevated CD4+/CD25+ Treg cell percentage in BCL patients when compared to control ones (31.1%±4.6% vs. 9.2%±1.1%, $P<0.05$). CR patients, however, had a cell percentage at 21.3%±3.3%, which was lowered than primary diagnosed patients but higher than healthy individuals. Those results suggest the elevated expression of Treg cells in BCL.

*TGF-β1 expression levels*

Both mRNA and protein levels of TGF-β1 were analyzed from peripheral blood samples by RT-PCR and ELISA, respectively. As shown in **Figure 2A**, mRNA level of TGF-β1 was significantly elevated in lymphoma patients compared to control ones ($P<0.05$). After treatment, however, mRNA level was decreased ($P<0.05$). Further ELISA for serum TGF-β1 protein levels

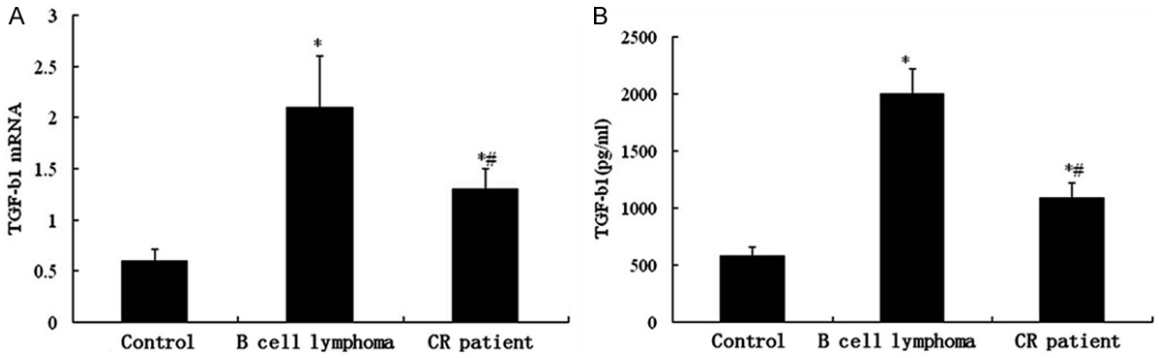


**Figure 2.** TGF-β1 expression in B cell lymphoma patients. A. mRNA level by RT-PCR; B. Serum protein level by ELISA. *, *P*<0.05 compared to control group; #, *P*<0.05 compared to lymphoma group.

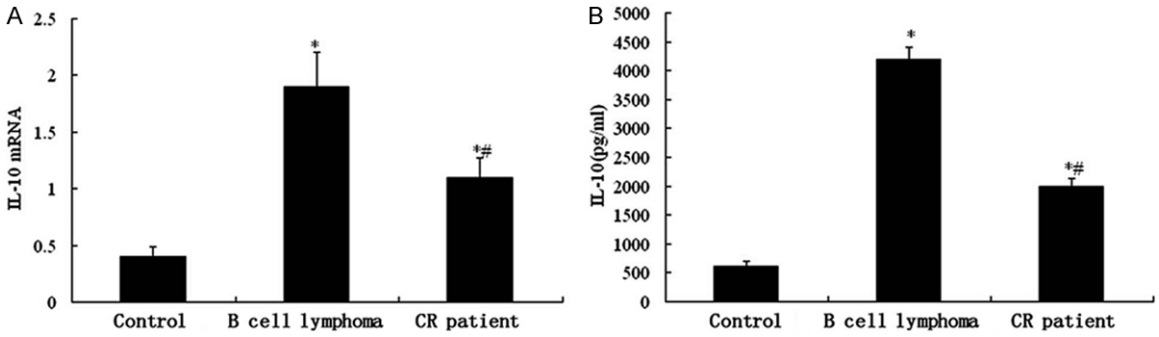


**Figure 3.** IL-10 expression in B cell lymphoma patients. A. mRNA level by RT-PCR; B. Serum protein level by ELISA. *, *P*<0.05 compared to control group; #, *P*<0.05 compared to lymphoma group.

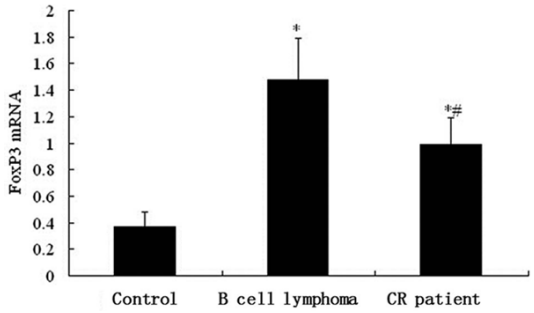


**Figure 4.** Foxp3 mRNA expression in B cell lymphoma patients. *, *P*<0.05 compared to control group; #, *P*<0.05 compared to lymphoma group.

obtained similar results (**Figure 2B**): it was potentiated in active lymphoma patients and was decreased in CR patients (*P*<0.05 in both cases).

*IL-10 expression levels*

We also investigated mRNA and protein levels of IL-10 in BCL patients and got similar patterns as those in TGF-β1. RT-PCR showed significantly elevated mRNA levels in active lymphoma patients, in addition to partial recovery in CR patients (**Figure 3A**, *P*<0.05). Protein levels of IL-10 had consistent patterns as those for mRNA (**Figure 3B**).

*Foxp3 mRNA levels in BCL*

We further investigated the expressional pattern of Foxp3 gene, which is a specific marker for Treg cells, in both lymphoma and controlled individuals. Results showed significantly elevated Foxp3 mRNA level in B-cell lymphoma patients who were at active stage (**Figure 4**, *P*<0.05). Patients at CR stage, however had significantly depressed Foxp3 mRNA levels compared to those at active stage (*P*<0.05).

## Discussion

As the most common non-Hodgkin lymphoma in adults, BCL had a relatively fast occurrence and progression, especially in diffuse large B cell lymphoma (DLBCL). BCL can occur at different age groups, and is heterogeneous in clinical prognosis due to its complicated symptoms, pathological features, genetics and molecular

biological mechanisms, all of which contribute to its high invasiveness and malignancy [15, 16]. Currently multiple treatment methods including surgery, combined chemotherapy and molecular target treatment have been widely used but did not significantly improve patient's life quality or their overall survival rates. Such difficulty is probably contributed by the inherent complexity of BCL pathogenesis and unclear pathogenic mechanism [17]. Therefore further studies regarding the mechanism governing pathogenesis and progression of BCL may benefit treatment efficacy, elongate patients' survival period and improve their life qualities.

Regulatory T cells, also named as CD4+/CD25+ Treg cells can maintain the dormant status of B cells via inhibiting parts of effector T cells and B cells, in addition to maintaining body immune homeostasis. Early studies indicated the crucial role of Treg cells in mediating autoimmune diseases [18, 19]. Recent studies suggested the involvement of Treg cells in immune tolerance of various malignant tumors including gastric, ovary and breast cancer, in addition to the inhibition of body's anti-tumor immune response [14]. This study found significantly elevated ratio of Treg cells in total PBMCs in BCL, and depressed Treg cell ratio in CR patients after treatment, suggesting the importance of those cells in facilitating occurrence and progression of BCL. Treg cells have dual functions in the sense of both maintaining body auto-immune tolerance by inhibiting T cell-mediated immune response against auto-antigens, and inhibiting anti-tumor immune response. They may also facilitate the progression of BCL via inhibiting activation and proliferation of CD8+ T cells to suppress anti-tumor immune response. All those functions of Treg cells require various combinations of cytokines [13]. We thus performed further studies in which major effector cytokines TGF-β1 and IL-10 were analyzed for their expression levels. Lymphoma patients had elevated expressions of both cytokines, which were depressed in CR patients, suggesting the possible involvement of those two immune-suppressing cytokines in Treg cell-mediated lymphoma pathogenesis.

As a highly-conserved specific marker for human Treg cells, Foxp3 played an important role in the development of CD4+/CD25+ Treg cells as its products maintain body immune homeostasis via inhibiting the production of active inducing cytokines [20, 21]. Foxp3-positive Treg cells exert a potent immune suppression function, making it a candidate gene for protecting Treg cells against immune surveillance during oncogenesis [22, 23]. This study found elevated expression of Foxp3 genes in BCL patients, further supporting the role of Treg cells in the occurrence and progression of BCL, in addition to the escape from immune surveillance and anti-tumor immune response.

In summary, elevated CD5+/CD25+ Treg cells existed in BCL patients, accompanied with higher Foxp3 gene expression. Our results showed that Treg cells may facilitate the oncogenesis and progression of BCL via immune suppressing cytokines.

## Disclosure of conflict of interest

None.

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