Frequency of IL-10-producing regulatory B cells associated with disease activity in thyroid-associated orbitopathy

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Abstract
● AIM: To investigate the association between IL-10-producing regulatory B (B10) cells and the clinical features of thyroid-associated orbitopathy (TAO).
● METHODS: A total of 30 patients with TAO were recruited at Zhongshan Ophthalmic Center from May 2015 to December 2015. Peripheral blood mononuclear cells (PBMCs) were separated from blood samples of 30 TAO patients and 16 healthy controls and stimulated with CD40 ligand and CpG for 48h. The frequency of IL-10⁺ B cells was examined by flow cytometry and the correlation between the frequency of IL-10⁺ B cells and clinical features of TAO was analyzed by SPSS.
● RESULTS: The frequency of IL-10⁺ B cells among CD19⁺ B cells in TAO patients was significantly lower than in healthy controls (TAO: 4.66%±1.88% vs healthy control: 6.82%±2.40%, P<0.01). The frequency of IL-10⁺ B cells showed a positive correlation with disease activity of TAO measured by Clinical Activity Score (CAS) (r=0.50, P<0.01), and became higher in TAO patients with family history of Graves’ disease (GD) (P=0.04).
● CONCLUSION: The decrease of the frequency of IL-10⁺ B cells in TAO patients indicates the deficiency of B10 cells in TAO, and the positive association with disease activity suggests its important role in TAO inflammation regulation.
● KEYWORDS: thyroid-associated orbitopathy; regulatory B cells; interleukin-10; flow cytometry

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INTRODUCTION

Thyroid-associated orbitopathy (TAO) is an autoimmune disease which potentially threatens vision, disfigures appearance and results in a pronounced loss of quality of life¹-². It is characterized by orbital T cell infiltration, release of proinflammatory cytokines and interaction with various cells including orbital fibroblasts through autoimmune mechanisms³-⁵. However, multiple lines of evidence suggest that B cells contribute to the pathogenesis of TAO by producing autoantibodies to the thyroid-stimulating hormone receptor (TSHR) and insulin-like growth factor-I receptor (IGF-IR), which leads to hyperthyroidism and potentially fibroblast activation, and have emerged as a therapeutic target⁶. Depleting CD20⁺ B cells with anti-CD20 antibody rituximab has shown potential benefit in controlling the disease activity of TAO⁷-⁸.

Regulatory B (Breg) cells are critical regulators of immune responses in autoimmune diseases⁹-¹¹. By producing suppressive cytokines interleukin-10 (IL-10) and transforming growth factor-β (TGF-β), Breg cells contribute to the maintenance of peripheral immune tolerance¹²-¹⁴. Analogous Breg cells in human could also suppress the differentiation of CD4⁺ T cells and secretion of interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α). These suppressive effects were mediated by IL-10¹⁰-¹¹,¹⁵. The functional IL-10-producing Breg cells are called B10 cells.

There are growing evidences that B10 cells play a key role in the pathogenesis of autoimmune diseases¹⁶-¹⁸. In this study, we evaluated the frequency of B10 cells in TAO patients according to the disease activity and severity.
SUBJECTS AND METHODS

Patients and Controls

Patients diagnosed with TAO at Zhongshan Ophthalmic Center from May 2015 to December 2015 were prospectively enrolled in this study. The diagnosis depended on the Bartley criteria[19]. Systemic examinations and orbital computed tomography (CT) or magnetic resonance imaging (MRI) scans were performed to exclude other confounding diseases (e.g. high myopia, orbital tumors, trauma, etc). Clinical Activity Score (CAS) was used to assess the disease activity in these patients[20-21]. The CAS score above 3/7 at the first examination or above 4/10 in successive examinations was defined as active[22]. The disease severity was evaluated according to the NOSPECS classification (no physical signs or symptoms, only signs, soft tissue involvement, proptosis, extraocular muscle signs, corneal involvement, and sight loss) reported by Werner[23]. Healthy controls with normal thyroid function [thyroid stimulating hormone (TSH): 0.27-4.20 μIU/mL] were recruited at the same time. Subjects with goiter, neoplastic, inflammatory, infectious diseases or other autoimmune disease were excluded from the study. This study adhered to the tenets of the Declaration of Helsinki and was approved by the Institutional Review Boards of Zhongshan Ophthalmic Center, Sun Yat-sen University. All participants provided written informed consent. Recorded demographic characteristics and clinical features of TAO patients are displayed in Table 1.

Sample Collection and Induction of B10 Cells

After collecting whole blood from the TAO patients and healthy controls, peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Histopaque-1077. Then PBMCs were washed with phosphate-buffered saline (PBS) solution twice and resuspended (2×10^6 cells/mL) in medium containing 10% fetal calf serum for culture. For B10 cell induction, PBMCs were stimulated with CpG (ODN 2006, 10 μg/mL; Invivogen), CD40 ligand (CD40L, 1 μg/mL; R&D Systems) or their combination for 48h at 37℃ as previously described[10]. For the last 5h, polymethyl acrylate (PMA; 50 ng/mL; Sigma-Aldrich-Aldrich), ionomycin (1 μg/mL; Sigma-Aldrich-Aldrich) and Brefeldin A (BFA; 1×solution/mL; BioLegend) (PIB) were added to increase intracellular protein expression and to stop intracellular cytokine secretion.

Flow Cytometry Analysis

For IL-10 detection, Fc receptors were blocked using Fc Block (BD Biosciences, USA). Cells were stained with the appropriate anti-CD19 antibody for 30min on ice. The cells were washed twice, fixed and permeabilized using a Cytofix/Cytoperm kit (BD Biosciences, USA) according to the manufacturer’s instructions and stained with anti-IL-10-PE (BioLegend, USA). All flow cytometry data were analyzed using FlowJo software.

Statistical Analysis

All graphs were prepared with GraphPad Prism 5.0. The t-test and analysis of variance were used to compare the levels of IL-10^+ B cells between groups. The correlation between clinical characteristics and the frequency of B10 cells was estimated by t-test and correlation analysis. P<0.05 was considered statistically significant. SPSS version 22.0 was used for all statistical tests (SPSS Inc.).
RESULTS

Demographic Characteristics and Clinical Features of Thyroid-associated Orbitopathy Patients
Totally 30 TAO patients and 16 healthy controls were enrolled in this study. The mean (±SD) ages of TAO patients and healthy controls were 39.77±12.69y (range, 19-64y) and 34.62±12.34y (range, 20-58y), respectively. Female of TAO patients and healthy controls were 16 (53.33%) and 10 (62.50%), respectively. There was no significant difference in gender distribution (P=0.78) and age (P=0.98) at sampling between TAO patients and healthy controls.

For 30 TAO patients, 8 cases (26.67%) had a family history of Graves’ disease (GD). Duration of TAO was 15.90±13.08mo (range, 1-48mo). Totally 18 patients were treated with systemic (40.00%) or local (20.00%) glucocorticoid, while 12 of the patients (40.00%) received no glucocorticoid treatment. A total of 20.00% of the TAO patients smoke. The mean CAS and NOSPECS score was 2.57±1.96 (range, 0-6) and 3.80±0.55 (range, 3-5), respectively. The mean free triiodothyronine (FT3), free thyroxine (FT4) and TSH was 4.94±1.71 pmol/L (3.15-10.50 pmol/L), 15.60±6.48 pmol/L (0.82-31.75 pmol/L) and 5.81±19.14 μIU/mL (0.00-102.00 μIU/mL), respectively. The demographic characteristics, clinical features and laboratory test of TAO patients were summarized in Table 1.

Percentage of B10 Cells Induced by Different Stimulations
PBMC from the blood of 16 healthy donors and 30 TAO patients were stimulated with CD40L, CpG, or with their combinations for 48h. Cells were treated with PIB for the last 5h to stop cytokine secretion, and B10 cells were detected by flow cytometry. Control group only treated with PIB for 5h in media induced negligible percentage of B10 (healthy individuals 1.48%±0.56%; TAO patients 1.00%±0.48%) among CD19+ B cells. Compared to control group, CD40L, CpG group, the combination of CD40L and CpG group induced a significantly higher percentage of B10 cells (P<0.01) in TAO and healthy individuals (Figure 1). So, we used the combination of CpG and CD40L to induce B10 cells in the following experiments.

Our results showed that the frequency of B10 cells among CD19+ B cells in TAO patients was significantly lower than
healthy individuals in the combination of CD40L and CpG group (TAO, 4.66%±1.88% versus healthy individuals, 6.82%±2.40%, \( P < 0.01 \); Figure 2).

**Association of B10 Cells with the Clinical Activity of Thyroid-associated Orbitopathy**  
Our results indicated that the frequency of B10 cells was significantly higher in TAO patients with family history of GD. When we compared active and inactive TAO patients, a significantly higher percentage of B10 cells was found in active TAO patients (Table 1). In addition, the TAO patients had a positive correlation between CAS and B10 cells frequency \( (r=0.50, P=0.004, \text{by correlation analysis; Table 2}) \). There were no differences in the proportions of B10 cells between patients with different clinical features in gender, smoking, glucocorticoid treatment, or NOSPECS (Table 1). The correlation of B10 cells frequency with age, disease duration, FT3, FT4, or TSH was not significant (Table 2).

**DISCUSSION**
In recent years, many studies have recognized the critical role of Breg cells in suppressing immune responses and maintaining peripheral tolerance by producing regulatory cytokines IL-10 (B10 cells). Our study indicated that the frequency of B10 cells was significantly associated with TAO disease activity assessed by CAS score, whereas the correlation with disease severity evaluated by NOSPECS was not significant. It suggests that the frequency of B10 cells may be a reflection of the disease activity in TAO patients. The clinical course of TAO involves two stages. After a progressive active phase characterized by inflammation and orbital tissue remodeling, the condition gradually stabilizes and eventually trends towards quiescence (inactive phase)\cite{24}. CAS is a validated scoring system designed to distinguish inflammatory from noninflammatory TAO\cite{21}. Based on the classical signs of acute inflammation (pain, redness, swelling, and impaired function), this scoring system was proposed as a clinical classification to discriminate easily between the active and quiescent stages of the disease and was modified in 1997\cite{22}. The sum of all points defines clinical activity: the score above 3/7 at the first examination or above 4/10 in successive examinations is defined as active\cite{22}. The frequency of B10 cells increased in active TAO patients and was significantly correlated with disease activity assessed by CAS score. B10 cells may contribute to the induction of regulatory T cells (Tregs) and down-regulate the inflammation\cite{25}. The underlying mechanisms between B10 cells and Tregs in TAO merit further investigation.

An increased frequency of Breg cells was also found in patients with systemic or organ-specific autoimmune diseases\cite{10,25-27}. However, other reports suggested a reduction in Breg numbers in rheumatoid arthritis\cite{17}, neuromyelitis optica\cite{28}, Graves’ disease and Hashimoto’s thyroiditis\cite{29} patients. The discrepancies between these studies may due to the disease activity of patients at test or the methods pulsing B cells for Breg detection.

This study has several limitations. The number of sample was small and there is no longitudinal follow-up. The frequency of B10 cells decreases in TAO patients and is associated with disease activity, and further studies should investigate the mechanism by which B10 cells contribute to the pathogenesis and disease activity of TAO.

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