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The expression of Bcl-6 in circulating follicular helper-like T cells positively correlates with the disease activity in systemic lupus erythematosus

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ABSTRACT

Increased circulating follicular helper-like T cells (cTfh) are reported in systemic lupus erythematosus (SLE) patients. However, whether B-cell lymphoma 6 (Bcl-6) is expressed in cTfh cells remains to be clarified. In this study, we found that the frequencies of $CD4^+CXCR5^{hi}PD\text{-}1^{hi}$cTfh, $CD4^+CXCR5^{hi}PD\text{-}1^{hi}ICOS^{hi}$, and $CD4^+CXCR5^{hi}PD\text{-}1^{hi}Bcl\text{-}6^+$ populations were significantly increased in SLE patients (n = 70) when compared with healthy controls (n = 48). Surprisingly, only $CD4^+CXCR5^{hi}PD\text{-}1^{hi}Bcl\text{-}6^+$ cTfh cells, rather than $CD4^+CXCR5^{hi}PD\text{-}1^{hi}$ population, were positively correlated with SLEDAI and anti-dsDNA antibodies. An elevated level of IL-21 was found in SLE $CD4^+$ T cells. Moreover, IL-21 promoted the enrichment of TET2 in Bcl-6 promoter region and induced Bcl-6 expression. Therefore, Bcl-6 expression in cTfh cells may represent a reliable marker for the disease activity in SLE.

1. Introduction

Systemic lupus erythematosus (SLE) is a systemic inflammatory autoimmune disease characterized by a loss of tolerance toward nuclear components, leading to an abundant autoantibody production and immune complex formation. SLE ultimately results in multiple organ and tissue damage, including damage to the skin, joints, vessels, kidneys and central nervous system [1]. The pathogenesis of SLE has not been well documented. However, multiple lines of evidence have shown that aberrant T and B cell activations contribute to the initiation and development of the disease [2,3].

Follicular helper T (Tfh) cells are a newly identified T-helper subset that specializes in stimulating GC formation and the selection of high-affinity B cells in GCs [4–6]. Due to their capacity to help B cells, Tfh cells are believed to contribute to abundant antibody production and thereby play a critical role in the pathogenesis of SLE. Tfh cells are distinguished by surface markers, such as CXCR5, PD-1, and ICOS, and the specific transcription factor, Bcl-6. CXCR5 guides Tfh cell migration into B-cell follicles, and ICOS delivers activation signals to $CD4^+$ T cells when these cells interact with antigen-presenting cells (i.e., dendritic cells and B cells) that express the ICOS ligand. ICOS signaling is essential for Tfh cell differentiation [7]. PD-1 is highly expressed in Tfh cells and serves as a negative regulator of Tfh cell differentiation [4–6,8] It has been reported that increased $CD4^+CXCR5^+$, $CD4^+CXCR5^{hi}PD\text{-}1^{hi}$ and $CD4^+CXCR5^{hi}PD\text{-}1^{hi}ICOS^{hi}$ populations in the peripheral blood are positively correlated with disease activity in SLE patients [9–12]. However, it remains unclear whether cTfh cells express Bcl-6 in SLE patients. Bcl-6 is a member of the BTB-zinc-finger family proteins and plays critical roles in various cell types of the innate and adaptive immune system. In T cells, Bcl-6 is essential for Tfh formation, provides help to B cells, and contributes to GC formation [13,14]. Bcl-6 has also been identified as the obligatory regulator for commitment through repression of other T cell lineages, including Th1, Th2, and Th17 cells [15,16].

In this study, we measured the percentage of $CD4^+CXCR5^{hi}PD\text{-}1^{hi}$, $CD4^+CXCR5^{hi}PD\text{-}1^{hi}ICOS^{hi}$, and $CD4^+CXCR5^{hi}PD\text{-}1^{hi}Bcl\text{-}6^+$ cells among PBMCs of SLE and healthy controls. Significantly increased frequencies of $CD4^+CXCR5^{hi}PD\text{-}1^{hi}$, $CD4^+CXCR5^{hi}PD\text{-}1^{hi}ICOS^{hi}$, and $CD4^+CXCR5^{hi}PD\text{-}1^{hi}Bcl\text{-}6^+$ cells were observed in SLE patients compared to normal controls, but not in RA patients. In addition, only the

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$CD4^+CXCR5^{hi}PD\text{-}1^{hi}Bcl\text{-}6^+$ percentage was positively correlated with the SLEDAI and anti-dsDNA antibody levels and negatively correlated with C4, even with the modified SLEDAI (without anti-dsDNA and complement). Noticeably, the elevated IL-21 level was found in lupus $CD4^+$ T cells, which was positively correlated with Bcl-6 expression and the addition of IL-21 can increase the expression level of Bcl-6. Furthermore, IL-21 was found to promote the enrichment of TET2 in Bcl-6 promoter region, indicating an epigenetic modulatory role of IL-21 in Bcl-6 induction. Together, these findings demonstrate that Bcl-6-expressing cTfh cells could be considered as a reliable marker of disease activity in SLE.

2. Materials and methods

2.1. Patients and controls

This study was approved by the ethics committee of the Second Xiangya Hospital, Central South University. A total of 70 SLE patients who fulfilled at least 4 of the SLE classification criteria of the American College of Rheumatology were recruited from outpatient clinics in the Second Xiangya Hospital of Central South University [17]. Lupus disease activity was assessed using the SLE Disease Activity Index (SLEDAI) [18]. A total of 48 sex- and age-matched healthy controls were recruited from the medical staff at the Second Xiangya Hospital and Changsha Blood Center. Written informed consent was obtained from all of the subjects. Patient characteristics, such as demographic data, cumulative clinical features, serological profile, and medications, were retrieved from the medical records. A physical examination and laboratory investigations, including the complete blood count; liver and renal functions; and levels of the anti-dsDNA antibody, ANA, and its complements C3 and C4, were performed at the study visit. Active lupus disease was defined as SLEDAI >4. Renal involvement was defined as significant proteinuria >500 mg/24 h, active urinary sediments, and/or a renal biopsy-demonstrated lupus nephritis at the time of the study. Hematological involvement was defined as the presence of autoimmune hemolytic anemia, leukopenia with $<4.0 \times 10^9$/l white blood cells, or thrombocytopenia with $<100.0 \times 10^9$/l platelets.

2.2. Flow cytometry

To examine the expression of surface markers and intracellular molecules, cells were incubated with a FcR blocking reagent (Miltenyi, Germany) for 10 min followed by primary antibodies, on ice, in the dark for 30 min. The antibodies used for the surface marker analysis included anti-human CD4-FITC, CXCR5-PE, and PD-1-PE-cy7 (BD Pharmingen, USA). For intracellular staining, cells were cytofixed and cytopermed using the Cytofix/Cytoperm Plus Kit (BD Pharmingen, USA) and stained with the intracellular antibody Bcl-6-APC (BD Pharmingen, USA) and IL-21-PE (BD Pharmingen, USA) for an additional 30 min, on ice, in the dark. Data were acquired by flow cytometry (BD, Canto II, USA) and analyzed using FlowJo (Tree Star, USA).

2.3. Cell sorting

PBMCs were separated from the peripheral blood of healthy controls and SLE patients by density gradient centrifugation (GE Healthcare, Switzerland). $CD4^+$ T cells were isolated by positive selection using Miltenyi beads according to the manufacturer's instructions (Miltenyi, Germany). Cells were incubated with a FcR blocking reagent (Miltenyi, Germany) for 10 min followed by primary antibodies, on ice, in the dark for 30 min. The antibodies used for the surface marker analysis included anti-human CXCR5-PE, and PD-1-PE-cy7 (BD Pharmingen, USA). Then cells were resuspended in 2 ml PBS and ready for cell sorting (FACSJazz, BD, USA).

2.4. Confocal microscopy

$CD4^+CXCR5^+PD\text{-}1^+$ cells were sorted and fixed using the Cytofix/Cytoperm Plus Kit (BD Pharmingen, USA) for intracellular staining of rabbit anti-human Bcl-6 (Cell signaling, USA) or mouse anti-human IL-21 (BD Pharmingen, USA) and Alexa Fluor® 594 conjugate Donkey anti-Rabbit IgG Secondary Antibody (Life USA) or Alexa Fluor® 488 conjugate secondary antibody Goat anti-mouse IgG Secondary Antibody (Life, USA) on ice and in the dark for 1 h. Then cells were analyzed by Zeiss LSM 780 (Zeiss, Germany).

2.5. Cell isolation, culture and differentiation

$CD4^+CDRA^+/RO^-$ naïve T cells were isolated by negative selection by Miltenyi beads according to the manufacturer's instructions. The purity of the enriched subset was validated by flow cytometry and was generally higher than 95%.

Tfh cell differentiation: $CD4^+CDRA^+/RO^-$ naïve T cells were seeded in anti-CD3 antibody (1 μg/ml) pre-coated 24-well plates (2.5×10^5 cell/ml) with or without NaCl in the presence of anti-CD28 antibody (1 μg/ml), IL-6 (5 μg/ml), TGF-β (1 μg/ml), IL-1β (10 μg/ml) and IL-23 (10 μg/ml) for 7 days. The medium was refreshed on Day 3 and Day 5 [19]. The cells were harvested for subsequent analysis.

In some experiments, IL-6, TGF- β, IL-1β, IL-12p70, IL-21 and IL-23 (20 ng/ml) were added to cells separately, in the presence of anti-CD3 and CD28 stimulation, for 5 days. The medium was refreshed on Day 5 and cells were harvested for subsequent analysis.

2.6. Quantitative PCR

Total RNA was extracted from cells using Trizol reagent (Invitrogen, USA). The sample was reverse-transcribed with the PrimeScript®RT reagent kit with gDNA Eraser (TaKaRa Biotech Co., China) using 1 μg of total RNA according to the manufacturer's instructions. The reaction mixture contained 2 μl of cDNA, 10 μl of SYBR Premix Ex Taq™ (TaKaRa Biotech Co., China), and 400 nM sense and antisense primers to a final volume of 20 μl. Transcripts were measured using a Rotor-Gene3000 (Corbett Research, NSW, Australia) thermocycler. The quantity of gene expression was calculated using the $2^{-\Delta Ct}$ methods and normalized to beta-actin. Primers for Bcl-6, IL-6, TGF-beta, IFN-γ and IL-21 were purchased from Life Technologies, USA.

Table 1
Clinical manifestations and clinical features of SLE patients at the time of the study.

Characteristics	SLE, n = 70
Age, years	33.71 ± 11.9
Female: male, no.	66: 4
Clinical manifestations, no. (%)	
Malar rash	16 (22.9)
Discoid rash	8 (11.4)
Photosensitivity	5 (7.1)
Oral ulcer	2 (2.8)
Arthritis/arthralgia	13 (18.5)
Autoimmune hemolytic anemia	15 (21.4)
Leukopenia	7 (10.0)
Lymphopenia	16 (22.8)
Immune thrombocytopenia	8 (11.4)
Renal involvement	39 (55.7)
Neurological involvement	1 (1.4)
SLEDAI	9.3 ± 7.1
Active disease, SLEDA >4 (%)	75
Serological features (%)	
ANA	96.4
Anti-dsDNA Abs, > +	46.0
Serum C3 < 80 mg/dl	62.0
Serum C4 < 20 mg/dl	79.0
Medications (%)	
Prednisolone or methylprednisolone	100.0
Hydroxychloroquine	83.3
Cyclosporine-A	11.9
Mycophenolate mofetil	10.5