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Role of the frequency of blood CD4$^+$ CXCR5$^+$ CCR6$^+$ T cells in autoimmunity in patients with Sjögren's syndrome

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ABSTRACT

The blood CD4$^+$ CXCR5$^+$ T cells, known as "circulating" Tfh, have been shown to efficiently induce naïve B cells to produce immunoglobulin. They play an important role in certain autoimmune diseases. In the present study, we show for the first time that the frequency of CD4$^+$ CXCR5$^+$ T cells is increased in pSS patients and positively correlated with autoantibodies in the blood. The concentration of Th17-like subsets (CD4$^+$ CXCR5$^+$ CCR6$^+$) in pSS patients was found to be significantly higher than in healthy controls. Functional assays showed that activated Th17-like subtypes in the blood display the key features of Tfh cells, including invariably coexpressed PD-1, ICOS, CD40L and IL-21. Th17 subsets were found to highly express Bcl-6 protein and Th1 and Th2 were not. Bcl-6 is believed to be a master transforming factor for Tfh cell differentiation and facilitate B cell proliferation and somatic hypermutation within the germinal center. These data indicate that Th17 subsets of CD4$^+$ CXCR5$^+$ T cells in the blood may participate in the antibody-related immune responses and that high frequency of CD4$^+$ CXCR5$^+$ CCR6$^+$ Tfh cells in blood may be suitable biomarkers for the evaluation of the active immune stage of pSS patients. It might provide insights into the pathogenesis and perhaps help researchers identify novel therapeutic targets for pSS.

1. Introduction

Primary Sjögren's syndrome (pSS) affects almost 0.5% of the general population and is second in prevalence only to rheumatoid arthritis among systemic autoimmune diseases [1]. One of the serologic hallmarks of the disease is the presence of variety of characteristic circulating autoantibodies, such as antinuclear antibodies, anti-Ro/SSA, and anti-La/SSB antibodies that mark the decline of the salivary glands, most of which relate to the -autoimmunity-mediated destruction of acinar cells [2]. Accordingly, B cell hyper-activation is a predominant feature of pSS related to hypergammaglobulinemia and to the production of autoantibodies [3]. Most studies on the subject have shown that autoantibodies play a decisive role in the pathogenesis of pSS but their creation and regulation in pSS remains unclear [4–6]. Thus, elucidating the mechanisms of autoantibody generation and regulation is crucial for the study of autoimmune diseases.

Physiologically, antibody responses including autoantibody are largely dependent on the help provided by CD4$^+$ T cells, which are fundamental for the generation of germinal centers (GC), a discrete structure in secondary lymphoid organs where selection of high-affinity B cells and development of B cell memory occur [7]. T follicular helper cells (Tfh) have recently emerged as a separate CD4$^+$ T helper lineage specialized in the assistance of B cells during GC reactions in secondary lymphoid tissue [8]. Tfh cells arise from activated T cells that express Bcl-6, a master transcription factor for Tfh cell differentiation [9]. The distinguishing features of Tfh cells are the expression of CXCR5, PD-1, ICOS, and CD40L and the secretion of IL-21.These molecules promote growth, differentiation, and class switching of B cells in the absence of Blimp-1 [10–12]. Recently, several studies have shown that over representation of Tfh cells is associated with the development of systemic autoimmunity, including RA and systemic lupus erythematosus (SLE) in human and animal models [13,14]. However, their association with pSS remains largely unknown.

In humans, most of the Tfh cells are located in the light zone of GC in secondary lymph nodules [15]. One problem that has arisen in studies of human Tfh cells, particularly in the investigation of patients suffering from autoimmune conditions, is that, the lymphoid tissue of these patients cannot be accessed. This makes it very difficult to identify Tfh cells and determine whether the generation or function of these cells is dysregulated. This has created a

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strong need to establish surrogate strategies to assess the quality of Tfh cell responses in humans. For this reason, researchers have used enumeration of circulating $CD4^+$ $CXCR5^+$ T cells in blood as a counterpart to measure Tfh cell disorders. Some types of $CD4^+$ $CXCR5^+$ T cells are known as "circulating" Tfh, which share functional properties with Tfh cells and appear to represent their circulating memory compartment [16]. Several studies have reported increased numbers of $CD4^+$ $CXCR5^+$ cells in the blood of patients with autoimmunity and decreased numbers in patients with antibody deficiencies [16–19]. This suggests that the levels of these cells may be a correlated with Tfh cells. However, little is known about the frequency of circulating Tfh cells in pSS patients [20]. In this study, we explored the frequency of circulating Tfh cells in human peripheral blood from pSS patients and examined the potential association of the frequency of $CD4^+$ $CXCR5^+$ T cells with laboratory measures. Progress in this field would be useful not only to defining the mechanisms that control autoimmune disease but also to identifying key pathogenic steps that could be targets of therapeutic action.

2. Material and methods

2.1. Patients and samples

Participants in this study were selected from patients seen at the Department of Clinical Immunology, Xi-jing Hospital, Fourth Military Medical University. Twenty-five pSS patients fulfilling the 2002 American-European consensus group criteria for pSS (55.6 ± 6.2 years old, mean ± s.d. $n = 25$) provided written informed consent and blood samples. The details of the clinical characteristics, and clinical lab data such as immunoglobulin, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) are determined. Disease activity was evaluated using the EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI) [21,22]. Twenty-five healthy volunteers were included as controls. Procedures were approved by the hospital Ethics Committee (No. 20080814–3) and performed after the provision of written informed consent.

2.2. Isolation of peripheral blood mononuclear cells (PBMC)

The samples were collected into collection tubes containing 0.2 ml sodium heparin. PBMC were obtained by Ficoll-Hypaque (FH) centrifugation of heparinized blood and resuspended to 1×10^6/ml in RPMI tissue culture medium containing 10% fetal calf serum (GIBCO, Grand Island, U.S.), streptomycin and penicillin (RPMI-FCS). PBMC cultures were grown in round-bottomed 5 ml polystyrene tubes (Falcon) containing 2×10^6 PBMC suspended in 2 ml RPMI-FCS [23].

2.3. Surface and intracellular staining and flow cytometry

Human PBMCs at 10^6/tube were stained in with both FITC-CXCR5 and PerCP-CD4, PE-CCR6, APC-CXCR3, APC-CD40L, APC-ICOS APC-PD-1, FITC-CCR7 or with isotype-matched control IgG (BD PharMingen San Diego, CA, U.S.) at room temperature for 30 min. After being washed with PBS, the cells were subjected to flow cytometry analysis using a FACS Calibur (Beckton Dickinson) and FlowJo software (v5.7.2). The cells were gated on the forward scatter of living cells and then centered on $CD4^+$ $CXCR5^+$ T cells. Then, the numbers of subtype of Tfh cells were determined by flow cytometric analysis. At least 50,000 events per sample were analyzed. Intracellular staining was performed with BD Phosflow Lyse/Fix buffer, BD Phosflow Perm/Wash buffer and PE-Bcl-6, APC-IL-21, Percp-IL-17 antibody according to the manufacturer's instructions [24].

2.4. Enzyme-linked immunosorbent assay detection of plasma immunoglobulin, anti-SSA, and anti-SSB

Serum immunoglobulin (Ig) levels, anti-Ro/SSA, and anti-La/SSB antibodies of IgG isotype in individual patients and HC were determined by commercial enzyme-linked immunosorbent assay (ELISA) as described (Euroimmun, Lübeck, Germany). Results were expressed in relative unit (RU) in accordance with the manufacturer's instructions. All samples were measured in duplicate, and the concentrations were calculated from a standard curve according to the manufacturer's protocol. Values above 10 RU were considered positive.

2.5. MACS® cell separation and T cell coculture

$CD4^+$ $CXCR5^+$ $CCR6^+$ T cells were isolated from the PBMC using an autoMACS to >95% purity as described. MACS® Cell Separation Reagents contained CD4-perCP, CXCR5-FITC, and CCR6-PE MicroBeads. $CD4^+$ T cells were negatively selected with a $CD4^+$ T-cell isolation kit (MiltenyiBiotec, Germany), yielding populations of $CD4^+$ cells with the 96–99% purity [25]. Next, $CXCR5^+$ $CCR6^+$ T cells were separated from $CD4^+$ T cells on the AutoMACS in two repetitive separation steps using a double positive selecting kit (MilltenyiBiotec). Cell separation and coculture with PHA was performed according to the manufacturer's instructions.

2.6. Real-time RT-PCR

Total RNA was extracted from Th17-like subtype of blood Tfh cell using Trizol(Invitrogen) according to the manufacturer's instructions. The concentration and the purity of RNA were determined by absorbance at 260/280 nm, and cDNA was synthesized using PrimeScript RT reagent Kit (TaKaRa). The primer sets used have been described in detail previously [26]. Real-time PCR was set up with Roche Probes Master reagents and Universal Probe Library hydrolysis probes. The expression of each gene was normalized to housekeeping gene ACTB. Relative levels of gene expression were measured using quantitative RT-PCR with SYBR Green master mix reagent in an ABI PRISM 7000 sequence detection system according to the manufacturer's instructions.

2.7. Statistical analysis

All statistics were performed using the SPSS version 17.0 software. The data are presented as the mean values ± standard deviations. The significance of the difference between groups was analyzed using one-way ANOVA testing with Bonferroni correction. The relationship between each two variables was evaluated using the Spearman rank correlation test. A two-sided $P < 0.05$ was considered statistically significant.

3. Results

3.1. High frequency of $CD4^+$ $CXCR5^+$ $CCR6^+$ T cells in the peripheral blood of pSS patients

To determine the frequency of $CD4^+$ $CXCR5^+$ T cell in peripheral blood, 25 pSS patients and 25 healthy subjects were recruited. As expected, the levels of serum anti- Ro/SSA, anti-La/SSB and RF in pSS patients were significantly higher than in healthy subjects(data not shown). To determine the potential role of peripheral Tfh cells

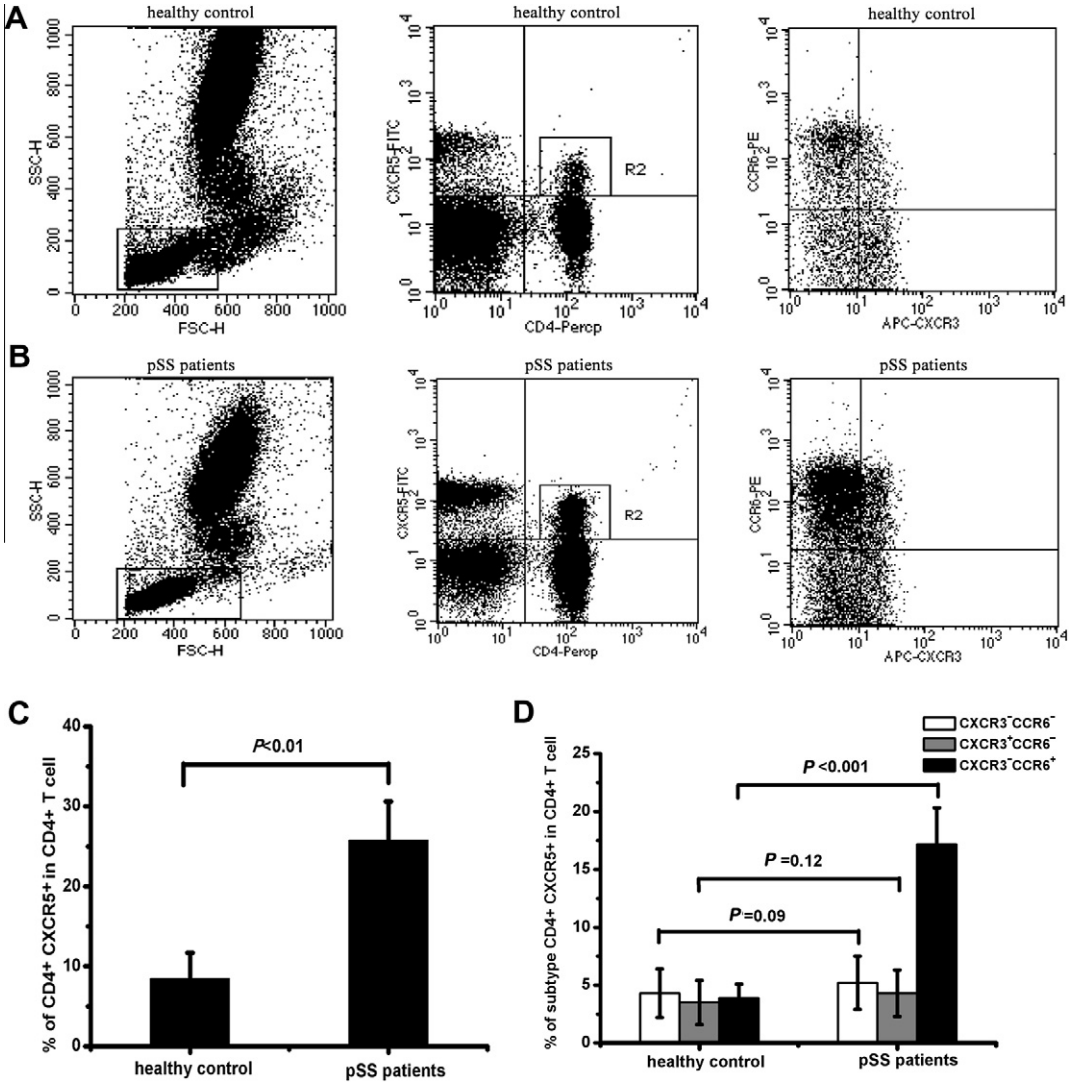

Fig. 1. FACS analysis of circulating Tfh cells. (A) The frequency of $CD4^+$ $CXCR5^+$ T cells and subtype analysis in healthy controls (HC). (B) The frequency of $CD4^+$ $CXCR5^+$ T cells and subtype analysis in pSS patients. (C) Relative prevalence of $CXCR5^+$ $CD4^+$ T cells among all CD4+ T cells. (D) Relative prevalence of subtypes (Th1, Th2, and Th17) cells among all $CD4^+$ T cells.

in pSS patients, the frequency of peripheral blood $CD4^+$ $CXCR5^+$ in $CD4^+$ T cell and the percentages of Th1($CXCR3^+$ $CCR6^-$) Th2 ($CXCR3^-$ $CCR6^-$) and Th17 ($CXCR3^-$ $CCR6^+$) in $CD4^+$ $CXCR5^+$ T cells were analyzed by flow cytometry (Fig. 1A and B). The percentages of $CD4^+$ $CXCR5^+$ T cells in pSS patients were 24.8 ± 4.8, significantly higher than in healthy individuals, who showed 8.6 ± 3.8 ($P = 0.001$, Fig. 1C). The frequency of the Th17 like subtype was significantly higher in pSS patients than in healthy individuals (Fig. 1D).

3.2. Blood $CD4^+$ $CXCR5^+$ $CCR6^+$ T cells correlated with autoimmunity in Patients with Sjögren's syndrome

We next determined whether the levels of blood $CD4^+$ $CXCR5^+$ $CCR6^+$ T cells were correlated with autoimmunity in patients with pSS. Clinical and laboratory variables including immunoglobulin, anti-Ro/SSA, anti-La/SSB, ESR, CRP, and ESSDAI were analyzed and frequency of $CD4^+$ $CXCR5^+$ $CCR6^+$ T cells were measured by flow cytometry. Spearman's correlation analysis revealed that the frequency of $CD4^+$ $CXCR5^+$ $CCR6^+$ Tfh cells was significantly positively correlated with the level of immunoglobulin, anti-Ro/SSA, anti-La/SSB and ESSDAI (Fig. 2C–F). However, no significant correlation was observed between the frequency of $CD4^+$ $CXCR5^+$ $CCR6^+$ T cells and ESR and CRP (Fig. 2A and B).

3.3. Expression of Tfh surface markers by stimulated $CD4^+$ $CXCR5^+$ $CCR6^+$ T cells from pSS patients

To further characterize $CD4^+$ $CXCR5^+$ $CCR6^+$ T cell subsets in the development of GC Tfh, we first determined whether the $CD4^+$ $CXCR5^+$ $CCR6^+$ T cells displayed the surface molecular signatures of Tfh cells, such as PD-1 ICOS CD40L. Flow cytometry showed that the frequency of $PD\text{-}1^+$, $ICOS^+$, and $CD40L^+$ cells in the Th17 subtype made up 25 ± 4.7, 22 ± 5.4, and 24 ± 3.4 percent, respectively, of the total number of $CD4^+$ $CXCR5^+$ T cells. This is much higher than the Th1 and Th2 subtypes (Fig. 3A–C). Collectively, these data suggest that blood $CD4^+$ $CXCR5^+$ $CCR6^+$ T cells in pSS patients continue to express the surfaces marker PD-1 ICOS and CD40L, but they express little CCR7 (Fig. 3D). This indicated that this subtype of circling Tfh cell displays the key features of Tfh cells after stimulation with PHA.

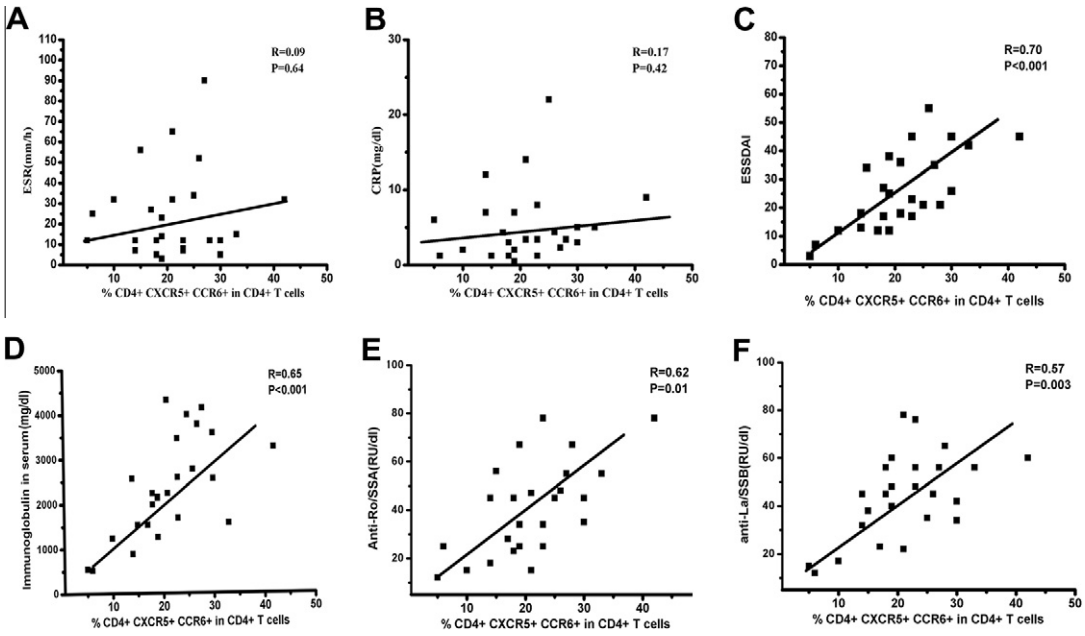

Fig. 2. Correlation of the levels of the clinical characteristics and clinical lab data with CXCR5$^+$ CD4$^+$ CCR6$^+$ Tfh cells in SS patients. (A) ESR. (B) CRP. (C) ESSDAI. (D) Immunoglobulin. (E) anti-Ro/SSA. (F) anti-La/SSB.

3.4. Intracellular IL-21 and Bcl-6 expression in CD4$^+$ CXCR5$^+$ CCR6$^+$ T cells after PHA stimulation in culture

We next evaluated the ability of native CD4$^+$ T lymphocytes to respond by increasing the synthesis of IL-21 and Bcl-6, which regulate Tfh cell differentiation. We analyzed the frequency of IL-21$^+$ and IL-17$^+$ among all CD4$^+$ CXCR5$^+$ CCR6$^+$ T cells using intracellular staining and flow cytometry. We also measured expression levels of mRNA encoding master regulators for different Tfh subsets. The data indicated that the frequency of IL-21$^+$ in Th17 subtype was higher than that of the Th1 or Th2 subtypes. Isolated CD4$^+$ CXCR5$^+$ CCR6$^+$ T cells from the blood showed upregulated IL-21 but not IL-17 suggesting that these subtypes are not likely to be Th17 subsets (Fig. 4A–C). When we looked at the master regulators of T-cell subsets, Th17 subtype T cells were found to be significantly up-regulated mRNA expression of Bcl-6 with the absence of Blimp-1 (Fig. 4D–F). These data suggest that blood CD4$^+$ CXCR5$^+$ CCR6$^+$ T cells in pSS expression IL-21 and Bcl-6, which indicate that Th17 subtypes of circling Tfh cells have the potential to differentiate into follicular helper T cells and to assist B cells.

4. Discussion

Although pSS has appeared to be a T-cell-mediated autoimmune disease over the past few decades, it is unclear which types of T cells are involved and how the dysregulation of these T cells triggers the development of pSS. Several studies show that patients with autoimmune diseases such as pSS or lupus display high-affinity somatically mutated auto antibodies in sera [27,28]. This suggests that Tfh cells are crucial regulators and are associated with the pathogenic processes of many autoimmune diseases in humans.

Tfh cells were not only found to offer powerful help to B cells with respect to the formation of GC in humoral immunity but also to induce B-cell-mediated systemic autoimmunity in humans. Several features of Tfh cells enable them to carry out these functions. Possibly the best-characterized B cell helper signal provided by Tfh cells is CD40L, which is a potent activator of B cells and can induce proliferation and, in combination with cytokines, isotype switching and differentiation of B cells [29,30]. Typically, Tfh cells are also identified by the coexpression of other surface markers, most commonly ICOS and PD-1. ICOS is a co-stimulatory molecule, and it plays an essential role in the development of Tfh cells and the delivery of helper signals to the B cells in vivo [31,32]. Furthermore, ICOS has been found to induce Bcl-6 expression, which is considered the main transcription factor for Tfh cell differentiation. More recently, evidence has shown that Bcl-6 facilitates the differentiation of CD4$^+$ T cells into Tfh cells through the inhibition of other key T helper cell transcription factors such as T-bet (Th1), GATA-3 (Th2), and RORγT (Th17) [33,34]. Similarly, PD-1 is highly expressed in Tfh cells. Several studies have suggested that PD-1 not only functions as a cell-surface phenotype marker but also has relevant functional implications, promoting B cell differentiation and somatic hypermutation [35,36]. Another important mechanism by which Tfh cells regulate B cell responses is through the secretion of cytokines. Tfh cells are characterized by expression of IL-21, a cytokine capable of modulating B cell differentiation, proliferation, and responsiveness to the GC reaction [37].

Although high expression of CXCR5 is one of the defining hallmarks of Tfh cells, CXCR5 is also expressed on peripheral blood human central memory CD4$^+$ T cells. It has been proposed that some of the blood CD4$^+$ CXCR5$^+$ T lymphocytes may represent the relevant Tfh cells involved in the GC reaction [16,17,38]. This is supported by human studies, where in the CD4$^+$ CXCR5$^+$ fraction in blood can be subdivided into CXCR3$^+$ Th1-like, CCR6$^+$ Th17-like, and CXCR3$^-$ CCR6$^-$ Th2-like Tfh cells.Th2- and Th17-like Tfh cells have been found to secrete IL-21, which could subsequently induce antibody production by naïve B cells.Th1-like Tfh cells did not express IL-21, and they could not support antibody production by B cells [16]. In this way, the simultaneous expressions of PD-1 on CXCR5$^+$ CD4$^+$ T cells in blood and the ability to express ICOS and produce IL-21 are helpful as markers of these circulating follicular T cells. Recent data have shown that the altered balance of Tfh cell subsets in the blood contributes to human autoimmunity. However, the definitive function of these cells is not fully understood and no such studies are available in pSS patients. Our study shows whether the frequency of CD4$^+$ CXCR5$^+$ T cells in the blood can be associated with active immunity in SS patients.

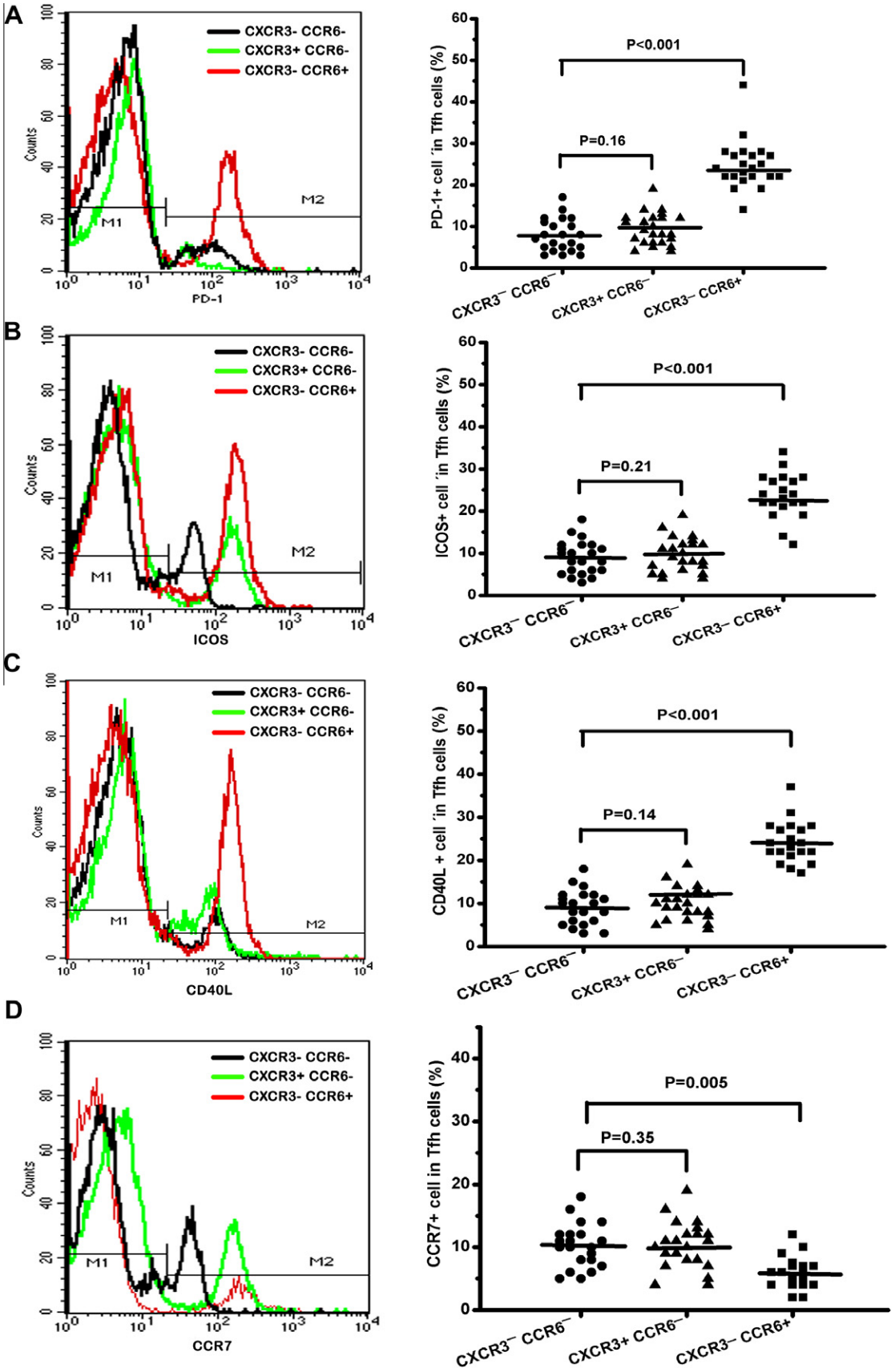

Fig. 3. Expression of Tfh surface markers by stimulated subtype of blood CD4+ CXCR5$^+$ T cells from pSS patients. (A) Frequency of PD-1$^+$ cells in subtypes of blood Tfh cells among total CD4$^+$ CXCR5$^+$ T cells. (B) Frequency of ICOS+ cells in subtypes of blood Tfh cells among total CD4$^+$ CXCR5$^+$ T cells. (C) Frequency of CD40L$^+$ cells in subtypes of blood Tfh cells among total CD4$^+$ CXCR5$^+$ T cells. (D) Frequency of CCR7$^+$ cells in subtypes of blood Tfh cells among total CD4$^+$ CXCR5$^+$ T cells.

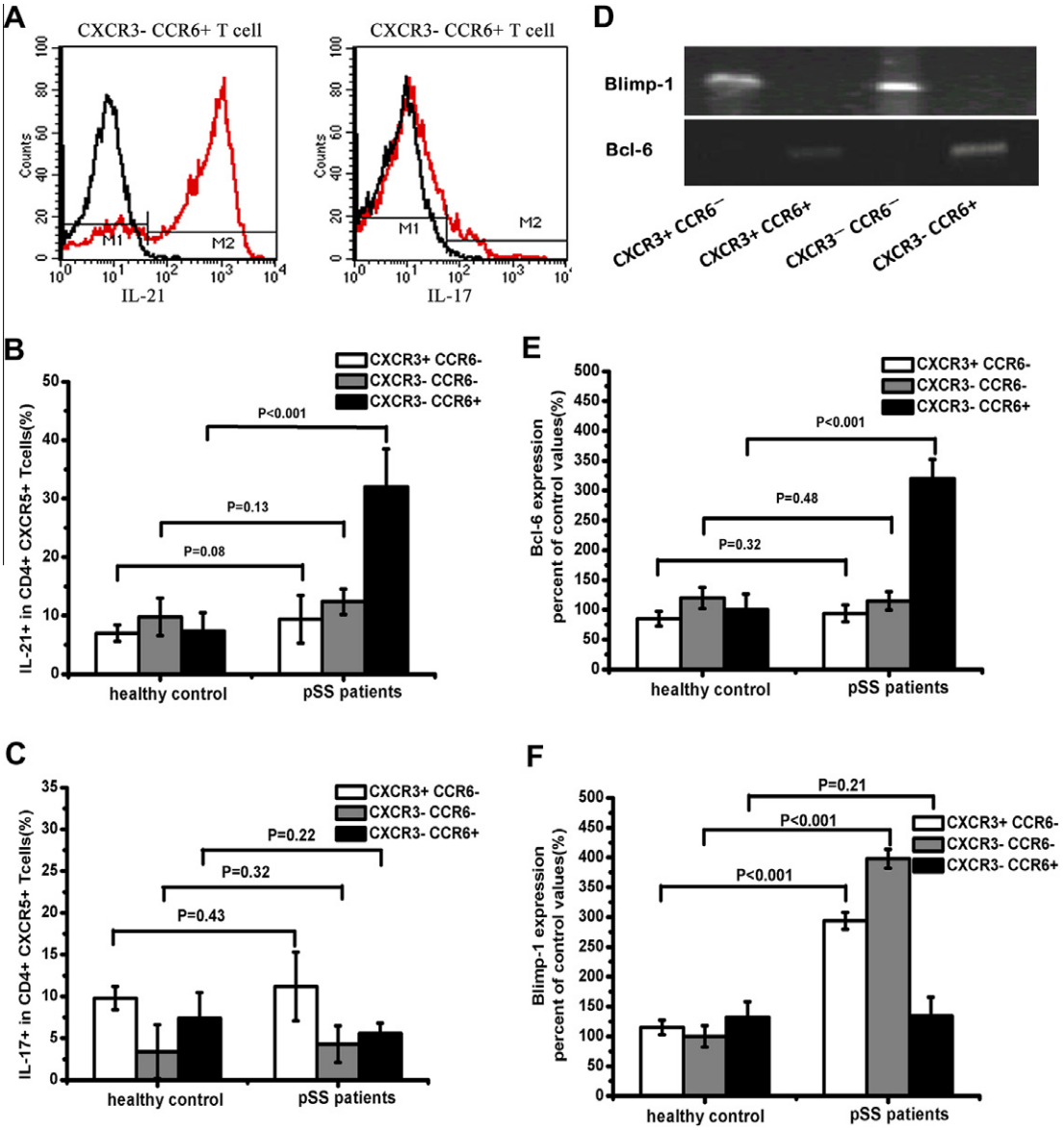

Fig. 4. Expression of each molecule after stimulation by PHA as analyzed by FACS and RT-PCR. (A) Expression of IL-21 or IL-17 gated to $CD4^+$ $CXCR5^+$ $CCR6^+$T cells. (B) Relative number of IL-21^+ cells in subtypes of blood Tfh cells among total $CD4^+$ $CXCR5^+$ T cells. (C) Relative number of IL-17^+ cells in subtypes of blood Tfh cells among total $CD4^+$ $CXCR5^+$ T cells. (D) Expression protein of transcriptional factor Bcl-6 and Blimp-1 in the subtype of blood Tfh cell populations was assessed by RT-PCR. (E) Expression of Bcl-6 mRNA was analyzed by real-time RT-PCR. (F) Expression of Blimp-1mRNA was analyzed by real-time RT-PCR.

The identification of functionally distinct Th cell subsets within blood $CXCR5^+$ compartment led us to consider that analysis might reveal dysregulation of Tfh cell responses in autoimmune diseases. For this reason, we analyzed blood levels of $CD4^+$ $CXCR5^+$ T cell subsets in samples from pSS patients and age-matched healthy controls. The results showed that the frequency of $CD4^+$ $CXCR5^+$ T cells was significantly higher in patients than in controls. The frequency of Th1 Th2 cells within the $CD4^+$ $CXCR5^+$ T cell compartment was not substantially different between the two groups. In contrast, the frequencies of Th17-like subtype cells within $CXCR5^+$ $CD4^+$ T cells were significantly higher in pSS patients than in healthy controls. The skewing of $CD4^+$ $CXCR5^+$ Th cell subsets resulted in a significant increase in the active stage over the inactive stage in SS patients. Functional assays showed that $CD4^+$ $CXCR5^+$ $CCR6^+$ subtype in blood invariably expressed PD-1, ICOS, and IL-21 and were closely associated with disease activity and serum autoantibody levels. Th17 subset, not Th1 or Th2, expressed Bcl-6 protein at high levels accompanied by repression of Blimp-1, which may be the master transcription factor for the differentiation of Tfh cells. This indicated that Th17 subsets of $CD4^+$ $CXCR5^+$ T cells could differentiate into Tfh and provide cognate help to antigen-specific B cells, leading to B cell proliferation and differentiation into antibody-producing plasma cells. These data indicate that Th17 subsets of $CD4^+$ $CXCR5^+$ T cells in the blood may participate in the antibody-related immune responses and that the high frequency of $CD4^+$ $CXCR5^+$ $CCR6^+$ Tfh cell subtypes in blood may be a biomarkers for the evaluation of active immune stage of SS patients. Our findings support the idea that autoantibody production is differentially regulated by different subsets of Tfh cells in pSS patients. It has emerged that some subtypes of blood circling Tfh cells are specialized to the assistance of B cells and the initiation and maintenance of GC reactions.

Further studies are required to determine the direct functional relevance of circling Tfh in regulating immune responses in pSS.

Such studies may prove highly rewarding because they might lead to new pathogenic insight into the processes underlying pSS. This might have important implications for the design of novel treatment target for autoimmune diseases.

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