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Original Article

$CD4^+CXCR5^+$ follicular helper T cells in salivary gland promote B cells maturation in patients with primary Sjogren's syndrome

Li Jin[1], Daliang Yu[1], Xiaomei Li[1], Ning Yu[1], Xiangpei Li[1], Yuanmin Wang[2], Yiping Wang[3]

[1]Department of Rheumatology and Immunology, Anhui Provincial Hospital Affiliated to Anhui Medical University, Hefei, Anhui 230001, China; [2]Centre for Kidney Research, Children's Hospital at Westmead, Westmead, The University of Sydney, NSW 2145, Australia; [3]Centres for Transplantation and Renal Research, Westmead Millennium Institute, The University of Sydney, NSW 2145, Australia

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Abstract: Objective: To examine amount of $CD4^+CXCR5^+$Tfh cells and B cells subsets in salivary gland and peripheral blood from patients with primary Sjogren's syndrome (pSS) and to analyze whether the frequency of $CD4^+CXCR5^+$Tfh cells is associated with pSS pathologic process. Methods: The percentages of $CD4^+CXCR5^+$Tfh cells and B cell subsets were examined by flow cytometry. B-lymphocyte chemoattraetant (BLC; also called CXCL13), IL-21, IL-6 from the serum of pSS patients was assessed by polymerase chain reaction–enzyme-linked immunosorbent assay (ELISA). Results: The percentages of $CD4^+CXCR5^+$Tfh cells in peripheral blood were increased in pSS patients, but decreased after treatment with glucocorticoid and/or immunosuppressive drugs. Abnormal B cell subsets appeared in salivary and peripheral blood of pSS patients. The frequency of salivary $CD4^+CXCR5^+$Tfh cells was positively correlated with $CD19^+CD27^+$ memory B cells and $CD19^+CD27^{high}$ plasma cells. Also increase of salivary $CD19^+CD27^{high}$ plasma cells was positively associated with serum ANA titer of pSS patients. Conclusions: $CD4^+CXCR5^+$Tfh cells are significantly increased in salivary and peripheral blood in pSS patients with aberrant $CD19^+CD27^+$ memory B cells and $CD19^+CD27^{high}$ plasma cells, suggesting that $CD4^+CXCR5^+$Tfh cells may contribute to the pathogenesis of pSS by promoting the maturation of B cells.

Keywords: Sjogren's syndrome, follicular helper T cell, B cell, germinal center

Introduction

Follicular helper T (Tfh) cells have been recently identified as a separate $CD4^+$T helper lineage, which are the specialized providers of help to B cells. It is one of the most studied subsets of $CD4^+$T cells in the last decade, which is primarily described in 2000, when numbers of groups reported a large proportion of $CD4^+$T cells in tonsils expressed chemokine receptor CXCR5 highly [1-3]. CXCR5 direct Tfh cell migration into B cell follicles in response to the specific ligand CXCL13 (B lymphocyte chemoattractant, BLC, B-cell–attracting chemokine 1, BCA-1) expressed by follicular dendritic cells (DCs) within the B-cell follicle [4, 5]. This colocalization of $CD4^+$T cell with B cells is pivotal to T-B interactions. Tfh cells also express inducible co-stimulator (ICOS), programmed death-1 (PD-1), and interleukin-21 (IL-21), which provide excellent markers for identification of Tfh cells and also serve important functions in their interactions with B cells [6, 7]. Interestingly, dysregulated function of Tfh cell has been reported in patients with autoimmune diseases, such as systemic lupus erythematosus (SLE), autoimmune thyroid disease (AITD) and systemic sclerosis, and some chronic disease such as chronic hepatitis B (CHB) [8-10]. However, little is known in patients with primary Sjogren's syndrome (pSS).

pSS is a chronic inflammatory autoimmune disease that affects the exocrine glands, mainly the salivary and lacrimal glands, but also frequently involve other exocrine glands, such as respiratory tract, gastrointestinal tract and skin, which are frequently accompanied by systemic symptoms [11-14]. The characteristic pSS histology is that T and B lymphocytes, dendritic cells (DCs) and macrophages infiltrate exocrine

Table 1. Patient Profiles

No. patient	Sex	Age (years)	Clinical and Biological Features	ESSDAI	Treatment	Steroid Dose (mg)
1	F	53	keratoconjunctivitis sicca, xerostomia, leucopenia, anemia hypergammaglobulinemia	6	HCQ,	10
2	F	61	keratoconjunctivitis sicca, xerostomia, leucopenia, arthralgia, decayed tooth	6	MTX	10
3	F	50	keratoconjunctivitis sicca, xerostomia, RTA, hypergammaglobulinemia	5	HCQ	15
4	F	45	keratoconjunctivitis sicca, xerostomia, arthralgia, decayed tooth, hypergammaglobulinemia	5	HCQ	10
5	F	58	keratoconjunctivitis sicca, xerostomia, decayed tooth, hypergammaglobulinemia	5	/	15
6	M	31	xerostomia, leucopenia, anemia, hypergammaglobulinemia	5	/	10
7	F	41	keratoconjunctivitis sicca, xerostomia, RTA, hypergammaglobulinemia	7	HCQ	15
8	F	59	keratoconjunctivitis sicca, xerostomia, hypergammaglobulinemia	4	TGP	/
9	M	75	keratoconjunctivitis sicca, xerostomia, hypergammaglobulinemia	5	TGP	/
10	F	25	xerostomia, leucopenia, anemia hypergammaglobulinemia	6	HCQ	10
11	F	27	keratoconjunctivitis sicca, leucopenia, RTA, hypergammaglobulinemia	8	HCQ	15
12	F	53	keratoconjunctivitis sicca, xerostomia, hypergammaglobulinemia	5	/	10
13	F	40	xerostomia, hypergammaglobulinemia	5	/	10
14	F	30	keratoconjunctivitis sicca, xerostomia, hypergammaglobulinemia	4	TGP	/
15	F	37	keratoconjunctivitis sicca, xerostomia, hypergammaglobulinemia	4	TGP	/
16	F	41	keratoconjunctivitis sicca, xerostomia, leucopenia, hypergammaglobulinemia	5	HCQ	10
17	F	47	keratoconjunctivitis sicca, xerostomia, leucopenia, anemia hypergammaglobulinemia	5	HCQ	10
18	F	23	keratoconjunctivitis sicca, xerostomia, leucopenia, anemia hypergammaglobulinemia	5	HCQ	10
19	F	28	keratoconjunctivitis sicca, leucopenia, anemia hypergammaglobulinemia	6	HCQ	10
20	F	71	arthralgia, decayed tooth xerostomia, leucopenia, anemia hypergammaglobulinemia	6	HCQ	10
21	F	78	keratoconjunctivitis sicca, xerostomia, leucopenia, hypergammaglobulinemia, decayed tooth	6	/	10
22	F	60	keratoconjunctivitis sicca, xerostomia, decayed tooth hypergammaglobulinemia	4	TGP	/
23	F	60	keratoconjunctivitis sicca, xerostomia, leucopenia, anemia hypergammaglobulinemia	7	HCQ	15
24	F	39	leucopenia, anemia, RTA hypergammaglobulinemia	8	HCQ	15

F, Female; M, Male; ESSDAI, EULAR Sjogren's Syndrome Disease Activity Index; HCQ, hydroxychloroquine; MTX, Methotrexate and TGP, total glucosides of paeony.

glands which impair the salivary and lacrimal glandular function and result clinically in xerostomia and keratoconjunctivitis sicca as well as high-titer serum autoantibodies and hypergammaglobulinemia. Nicholas Simpson *et al* have demonstrated increased circulating Tfh cells which were defined as $ICOS^{high}$ $CXCR5^{+}CD4^{+}$ or PD-1^{high} $CD4^{+}$ in SS patients [9]. Ectopic germinalcenterlike structure found in one fifth of patients with SS represents the histologic hallmark of this abnormal B cell proliferation [14].

In this study, we examined the frequency of Tfh cells and B cell subsets in peripheral blood and/or salivary gland from pSS patients, and explored possible correlation between abnormality of Tfh cells and pathogenesis of pSS.

Materials and methods

Study subjects

A total of 24 patients diagnosed as pSS were referred to the Department of Rheumatology and Immunology in Anhui Provincial Hospital from February 2011 to December 2011 and fulfilled the 2002 revised criteria established by the American-European Consensus Group [15] (**Table 1**) . Disease activity was evaluated using the EULAR Sjogren's Syndrome Disease Activity Index (ESSDAI). None of the patients enrolled were treated with glucocorticoid and/or immunosuppressive drugs. All patients of peripheral blood were collected. 20 healthy controls enrolled from Health Screening Center in Anhui Provincial Hospital. Salivary gland biopsies were obtained from 24 patients and controls (4 patients with xerostomia and/or eye drying who did not fulfill the 2002 revised criteria). The clinical laboratory data such as erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), immunoglobulin and clinical characteristics are determined. The research protocol was reviewed and approved by the Hospital Ethics Committee. Informed consent was obtained from all patients and controls.

Cell isolation

Peripheral blood mononuclear cells (PBMCs) from the patients and healthy controls were purified by density-gradient centrifugation on Ficoll-Hypaque centrifugation (1.077 density).

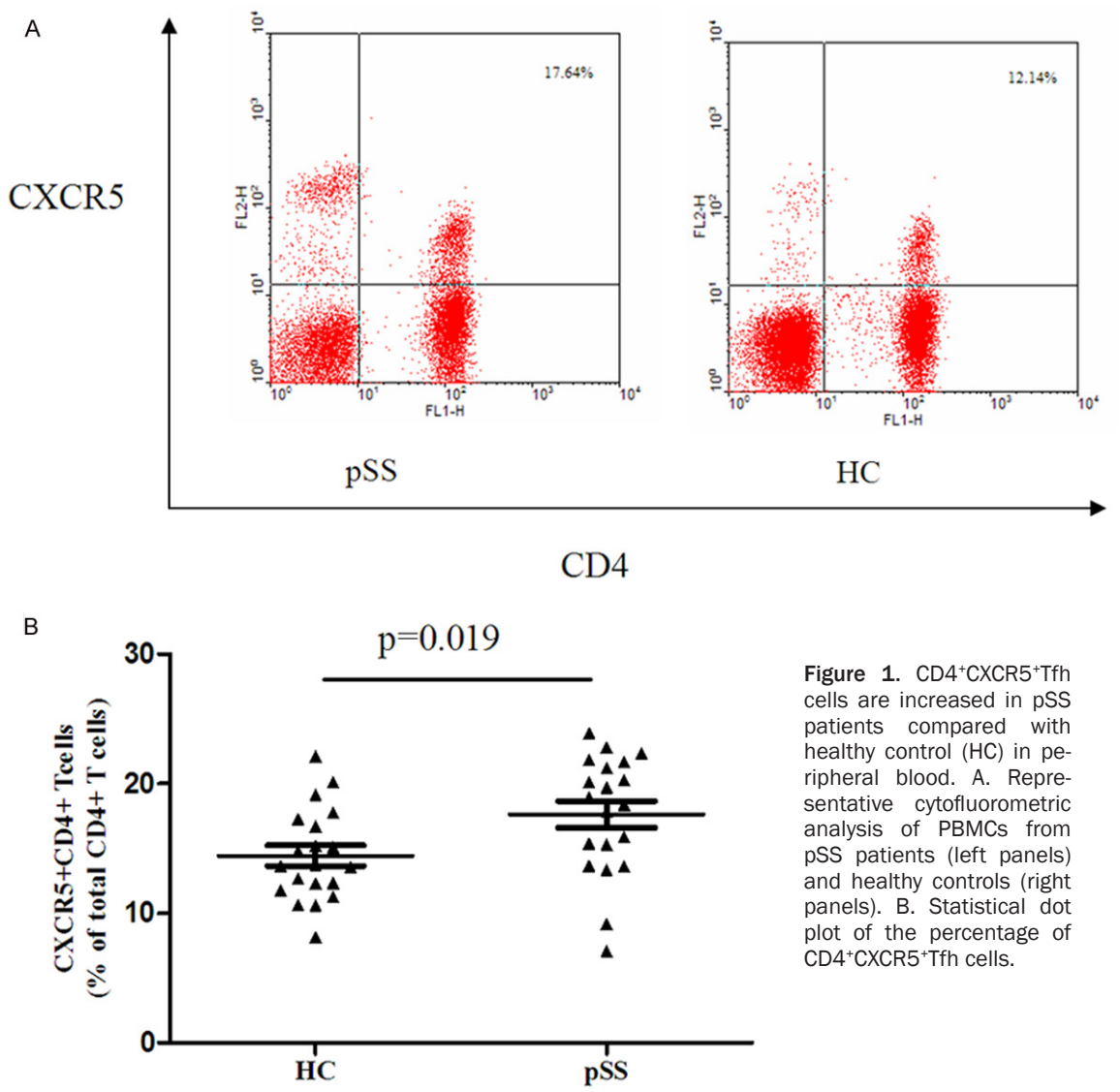

Figure 1. $CD4^+CXCR5^+$Tfh cells are increased in pSS patients compared with healthy control (HC) in peripheral blood. A. Representative cytofluorometric analysis of PBMCs from pSS patients (left panels) and healthy controls (right panels). B. Statistical dot plot of the percentage of $CD4^+CXCR5^+$Tfh cells.

The fresh salivary gland samples were obtained from patients with pSS and controls. Single cells were collected by mechanical disruption, then digested with 0.5% collagenase II (Sigma) at 37°C for 1.5 hours, and mashed gently using a plunger through a 70 um strainer to make single-cell suspensions. A fraction of single-cell suspensions was filtered through a 40 um strainer to make mononuclear cells. After washing, cells were centrifuged at 1800 rpm for 10 min and re-suspended. All cell collections were stained for flow cytometry.

Flow cytometry

Human PBMCs and salivary single cell at 10^6/tube were stained in duplicate with PerCP-anti-CXCR5 (Biolegend, San Diego, USA), FITC-anti-CD4, and APC-anti-CD27, Pecy5.5-anti CD19, or isotype-matched control IgG (Beckton Dickinson, San Jose, USA) at 4°C for 30 minutes, respectively. After being washed with PBS, the cells were acquired by FACSCalibur (Beckon Dickinson) and data were analyzed using FlowJo software (v5.7.2).

Enzyme-linked immunosorbent assay (ELISA)

Level of plasma BLC-1, IL-6 and IL-21 was done using ELISA for human BLC-1, IL-6, and IL-21 (Cusabio). All samples were analyzed in duplicate using the average of the optical density (OD) values to calculate concentrations.

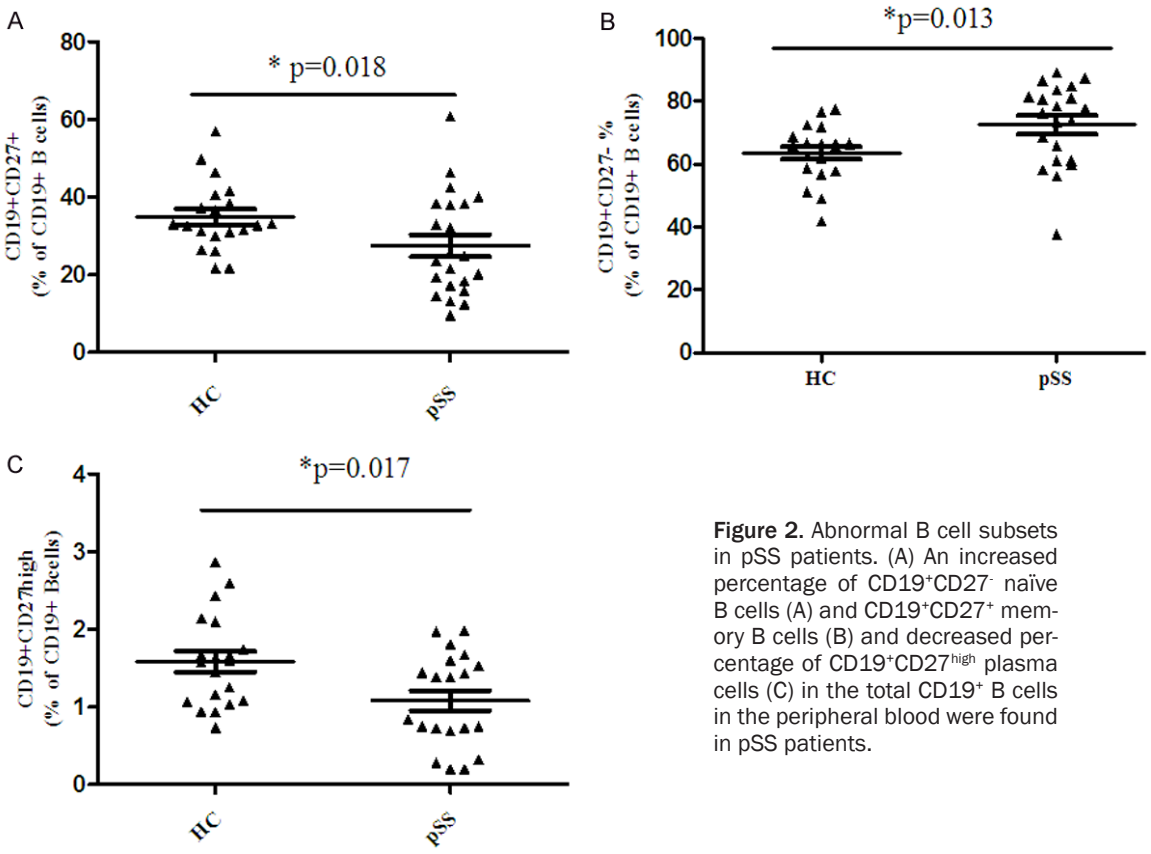

Figure 2. Abnormal B cell subsets in pSS patients. (A) An increased percentage of $CD19^+CD27^-$ naïve B cells (A) and $CD19^+CD27^+$ memory B cells (B) and decreased percentage of $CD19^+CD27^{high}$ plasma cells (C) in the total $CD19^+$ B cells in the peripheral blood were found in pSS patients.

Laboratory assessment

Serum antinuclear antibodies (ANA) were analyzed by indirect immunofluorescence assay on HEp-2 cell slides. Antibodies against Ro/SSA and La/SSB were detected in serum by immunoblotting. IgG, IgA, and IgM serum levels were measured by immunoturbidimetry assay.

Statistical analysis

Data were analyzed with SPSS version 13.0 software. The data were presented as the mean values ± standard deviations. The significance of the difference between groups was analyzed using independent T testing with Bonferroni correction. For correlation analyses, either Spearman's *r* or Pearson's *r* was calculated. A two-sided $P < 0.05$ was considered statistically significant.

Results

Expression of $CD4^+CXCR5^+$Tfh cells in the peripheral blood and salivary glands of pSS patients

In our study, we enrolled 24 pSS patients without treatment and 20 healthy controls to determine the percentage of $CD4^+CXCR5^+$Tfh cell (**Figure 1A**) in $CD4^+$T cells in peripheral blood. The frequency of $CD4^+CXCR5^+$T cell in pSS patients without treatment was significantly higher than that in healthy controls (17.90 ± 4.40% versus 14.45 ± 3.54%, $P = 0.019$, **Figure 1B**). At the same time, we detected the percentage of $CD4^+CXCR5^+$Tfh cells in $CD4^+$T cells in the salivary glands, which was 11.47 ± 6.23%. However, $CD4^+CXCR5^+$Tfh cells were rarely detected in salivary glands of controls.

Abnormal peripheral blood B cell subsets in pSS patients

We next examined the frequency of B cell subsets in peripheral blood from pSS patients and healthy controls. A significant reduction in the number of peripheral $CD27^+$ memory B cells and a increase in frequency of peripheral $CD27^-$ naive B cells were found in pSS patients [16, 17], $CD27^+$ memory B cells were decreased in pSS patients without treatment compared with healthy controls (26.49 ± 9.26% versus 34.90 ± 8.94%, $P = 0.018$, **Figure 2A**). The percentage of $CD27^-$ naïve B cells were higher than that in healthy controls (72.50 ± 12.80% versus

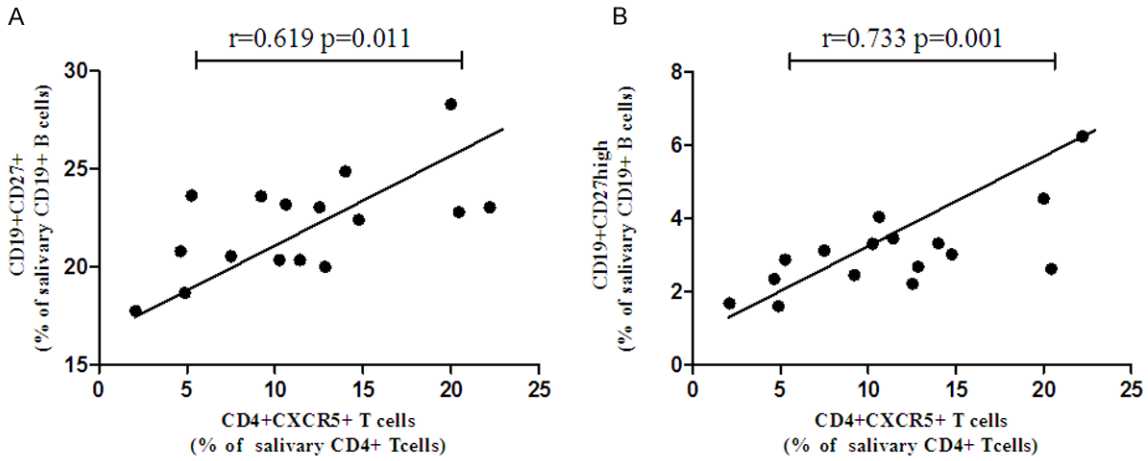

Figure 3. CD4$^+$CXCR5$^+$Tfh cells in salivary gland are positively correlated with memory B cells and plasma cells in untreated pSS patients.

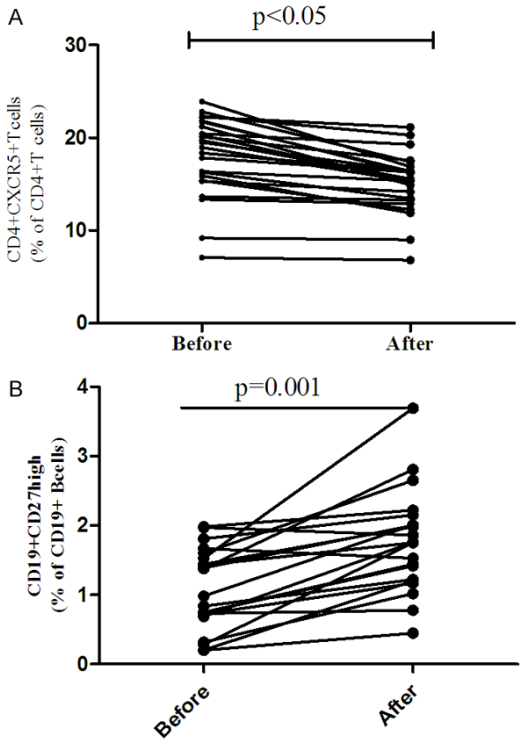

Figure 4. The influence of treatment to Tfh cells and B cell subsets in peripheral blood. A. Decreased percentage of CD4$^+$CXCR5$^+$Tfh cells in the peripheral blood after treatment for 4 weeks. B. Increased percentage of CD19$^+$CD27^{high} plasma cells in the peripheral blood after treatment for 4 weeks.

63.51 ± 8.95%, P = 0.013, **Figure 2B**). On the other side, we also found the decreased number of peripheral CD27^{high} plasma cells in pSS patients without treatment compared with healthy controls (1.12 ± 0.60% versus 1.58 ± 0.59%, P = 0.017, **Figure 2C**).

Correlation between CD4$^+$CXCR5$^+$Tfh cells and B cell subsets in pSS patients

The frequency of CD4$^+$CXCR5$^+$Tfh cells and B cell subsets were also examined both in the peripheral blood and salivary gland in pSS patients without treatment. However, no CD4$^+$CXCR5$^+$Tfh cells, CD27$^+$ memory B cells and CD27^{high} plasma cells were found in salivary glands of controls. We found that there was no correlation between CD4$^+$CXCR5$^+$Tfh cells with B cell subsets in peripheral blood. However, the frequency of CD4$^+$CXCR5$^+$T cells in salivary glands was positively correlated with the percentage of CD27$^+$ memory B cells (r = 0.619, P = 0.011, **Figure 3A**) and CD27^{high} plasma cells in peripheral blood (r = 0.733, P = 0.001, **Figure 3B**).

Change of the expression of CD4$^+$CXCR5$^+$Tfh cells and B cells subsets in pSS patients by treatment

The percentage of CD4$^+$CXCR5$^+$Tfh cells in peripheral blood was obviously reduced in pSS patients after treatment with glucocorticoid and/or immunosuppressive drugs (15.09 ± 2.88% versus 17.90 ± 4.40%, P = 0.037, **Figure 4A**), along with the increased frequency of CD27^{high} plasma (2.04 ± 0.92% versus 1.12 ± 0.60%, P = 0.001, **Figure 4B**). Whereas, there was no apparent influence to the frequency of

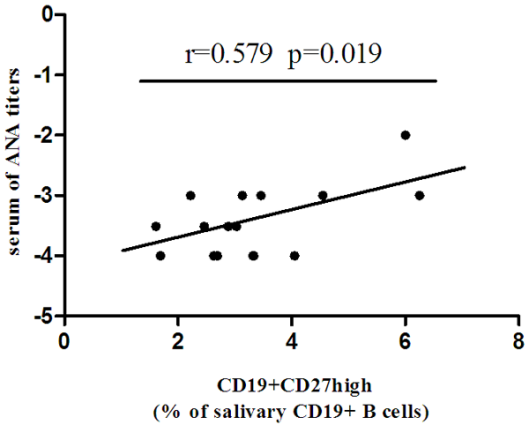

Figure 5. $CD19^{+}CD27^{high}$ plasma cells in salivary gland are positively correlated with serum ANA titers in untreated pSS patients.

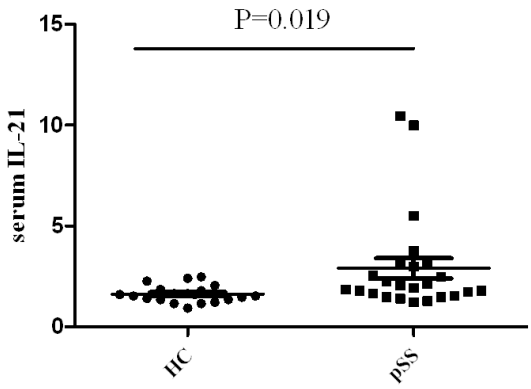

Figure 6. Serum IL-21 levels are increased in pSS patients compared with healthy control (HC).

$CD27^{-}$ naive B cells or $CD27^{+}$ memory B cells by treatment.

Relevance of $CD4^{+}CXCR5^{+}$Tfh cells and B cells subsets with laboratory parameter

We also explored the relevance of the frequency of $CD4^{+}CXCR5^{+}$Tfh cells and B cells subsets in the peripheral blood and salivary glands with clinical parameters. The frequency of $CD19^{+}CD27^{high}$ plasma cells in salivary gland are significantly correlated with serum ANA titers (**Figure 5**). However, no correlation has been found among $CD4^{+}CXCR5^{+}$Tfh cells, B cells subsets in the peripheral blood and clinical parameters including ESR, Ro/SSA and La/SSB, ANA titers.

Serum levels of related cytokines in pSS patients

Finally the serum cytokines in pSS patients without treatment were measured by ELISA. Serum IL-21 levels in pSS without treatment and healthy controls have shown a significant increase in pSS compared to healthy controls (2.86 ± 0.47, and 1.68 ± 0.42, $P < 0.05$ (**Figure 6**). However, the levels of serum BLC1, IL-6 in pSS patients have no significant change compared with healthy controls.

Dsicussion

Tfh cells are a key regulator in humoral immunity. The crucial role of these Tfh cells is helping B cell maturation and the production of antibodies in response to foreign antigens [18]. Previous studies have shown that characterized the frequency of peripheral Tfh cells in several autoimmune diseases [10, 19]. However, cellular and humoral mechanisms underlying pathogenesis of Sjögren's syndrome are still not fully understood. In this study, we demonstrated that salivary gland $CD4^{+}CXCR5^{+}$Tfh cells and abnormal B cells subsets significantly increased in pSS patients. The findings also clearly indicated that the percentage of $CD4^{+}CXCR5^{+}$Tfh cells in the salivary glands from pSS patients were correlated positively with $CD19^{+}CD27^{+}$ memory B cells and $CD19^{+}CD27^{high}$ plasma cells, yet the frequency of $CD19^{+}CD27^{high}$ plasma cells in salivary gland are significantly correlated with serum ANA titers.

Although the clinical symptoms of pSS are varied among patients and the pathogenetic mechanisms of this autoimmune disease have not been exactly elucidated. The characteristic histological feature of pSS is peri-ductal focal lymphocytic aggregates such as $CD4^{+}$T cells, B cells, plasma cells et al. Follicular dendritic cells (DCs) and proliferating B cells consist of germinal centerlike structures replace the secretory units [16, 20]. However, subsets of T cells participate in pathogenesis of pSS is still unknown.

Tfh cells are newly discovered $CD4^{+}$ effector helper T cell subsets, which have unique functions. According to the transcription factor and production of different cytokines, $CD4^{+}$ effector helper T cells can be divided into several subsets. Tfh cells expressed transcription factor

Bcl-6, and produced IL-21, for the sake of help B cells produce antibodies in humoral immune response, which are mostly located in the follicular area of lymphoid organs [21, 22]. Tfh cells were guided by CXCR5, a chemokine receptor, and localized to the germinal center. Tfh cells are the helper T cells for helping B cells to produce antibodies [23]. The basic function of Tfh cells involve B cell selection and differentiate into memory cells and plasma cells in the process of antibody affinity maturation [24]. These studies demonstrated that ectopic germinal center of non lymphoid tissues in many autoimmune diseases may be closely related to the pathogenesis [25]. The importance of function of Tfh cells in autoimmune diseases have been gradually recognized in recent years [26]. abnormal Tfh related molecules expression were found in several murine models of autoimmune diseases [27]. In 2010, Nicholas Simpson et al. found that elevated $CD4^+CXCR5^+$T cells accounted for the proportion of $CD4^+$T cells in the peripheral blood of SLE patients [9]. However, studies related to the role of Tfh cells are limited. We, for first time, reported salivary Tfh cells are increase abnormally and correlated to clinical parameter and treatment.

Results from our study showed that the frequency of $CD4^+CXCR5^+$T cells in $CD4^+$T cells in peripheral blood from untreated pSS patients were higher than that of healthy controls and decreased after therapy. This result is lined partly with the report by Zhu *et al* [28]. However, Zhu et al showed that the percentage of $CD4^+CXCR5^+$T cells was correlated to the titer of ANA in pSS patients, suggesting that $CD4^+CXCR5^+$T cells in peripheral blood may be participated in the occurrence of disease. Our results, displayed no correlation between the percentage of peripheral blood of $CD4^+CXCR5^+$Tfh cells and the titer of ANA and Ig. However, in the salivary glands, the percentage of $CD4^+CXCR5^+$Tfh cells was significantly correlated with $CD19^+CD27^+$ memory B cells and $CD19^+CD27^{high}$ plasma cells. Schaerli *et al* [2] have found that the majority of $CD4^+CXCR5^+$T cells in peripheral blood are $CD45RO^+$ memory T cells, with no expression of cell activation and costimulatory molecules (CD40, OX40, ICOS), in which way they do not participate in the instant immune response. Experiments in vitro showed unstimulated peripheral blood $CXCR5^+$ T cells cannot help co-cultured B cells produce antibodies. Therefore, they indicated $CD4^+CXCR5^+$ cells in the peripheral blood are in quiescent condition [29]. Due to $CD4^+CXCR5^+$T cells are not the true type Tfh cells same as that in salivary, it may explain why salivary $CD4^+CXCR5^+$ cells, but not peripheral $CD4^+CXCR5^+$ cells, have positive correlation with levels of ANA.

B cell hyper-activation is a predominant feature of pSS related to hyper-gammaglobulinemia and to the production of autoantibodies. The frequency of B cell subsets are varied among patients with pSS. A significant reduction in the peripheral $CD27^+$ memory B cells and increased frequency of peripheral $CD27^-$ naive B cells were found in pSS patients that is similar to results from other reports. We found that the frequency of $CD19^+CD27^{high}$ plasma cells in salivary gland are significantly correlated with serum ANA titers. Increase of salivary Tfh cells and B cell subsets contributed to the formation of the germinal centers, which is critical to pathogens of pSS.

In conclusion, we found a significant increase in frequency of peripheral blood $CD4^+CXCR5^+$Tfh cells and a decrease of B cell subsets in peripheral blood of pSS patients, which do not correlated to serum ANA titers. However, abnormal increase of $CD4^+CXCR5^+$Tfh cells and B cell subsets in salivary gland were significantly correlate to serum ANA titers, suggesting that salivary $CD4^+CXCR5^+$Tfh cells are the effector cells and may participate in pathogenesis of pSS.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Xiaomei Li, Department of Rheumatology and Immunology, Anhui Provincial Hospital Affiliated to Anhui Medical University, Hefei, Anhui 230001, China. E-mail: lixiaomei1@medmail.com.cn

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