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# Biomedical statistics study on the correlation between peripheral blood follicular helper T cell subsets and primary Sjogren's syndrome

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**ABSTRACT**

To study the expressions of follicular helper T cell (Tfh) subsets——Tfh1, Tfh2, and Tfh17, in peripheral blood (PB) of primary Sjogren's syndrome (PSS) patients in the active phase and remission period after treatment, and to analyze the pathogenic effects of Tfh subsets in PSS patients. The proportions of Tfh1, Tfh2, and Tfh17 were measured by flow cytometry in the healthy group, PSS patients group, active phase group, and remission phase group. Enzyme-linked immunosorbent assay was used to detect IL-21 expression in SS patients in active and remission stages. Biomedical statistics was used to analyze the correlation between Tfh subsets and SS disease activity index; and analyze the correlation between the proportions of Tfh subsets in the healthy group, primary group, active stage, and remission stage In contrast with the healthy group, the levels of Tfh1, Tfh2, and Tfh17 in CD4+ T cells of PSS patients were prominently reduced. PSS patients in the active phase had significantly lower Tfh1, Tfh2, and Tfh17 levels but remarkably higher IL-21 levels than in the remission phase. The contents of Tfh1, Tfh2, and Tfh17 are negatively correlated with the severity of PSS.

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## Introduction

Primary Sjogren's syndrome (PSS) is an autoimmune disorder with characteristics of progressive inflammation and damage to the lacrimal gland and salivary (1, 2). Follicular helper T cells (Tfh) cells are a distinct lineage of CD4 + T helper (th) cells that are essential for humoral immunity (3). Tfh as a group of helper T cell subsets newly discovered in recent years, characteristically express C-X-C Motif Chemokine Receptor 5 (CXCR5), Programmed Cell Death 1 (PD-1), and other molecules, which can be chemotactic to the germinal center of lymphoid follicles and help the biological function of B lymphocytes (4). CD4+ memory cells expressing CXCR5 in peripheral blood (PB) have functions similar to germinal center Tfh cells. According to the differential expression of their surface chemokine receptors, C-C Motif Chemokine Receptor 6 (CCR6) and C-X-C Motif Chemokine Receptor 3 (CXCR3), PB $CD4^+CD45RA^-CXCR5^+$ cells are grouped into three subgroups: Tfh1($CXCR3^+CCR6^-$), Tfh17($CXCR3^-CCR6^+$) and Tfh2($CXCR3^-CCR6^-$) (5).

Relevant experiments have confirmed that skewed distribution of Tfh cell subsets contributes to the pathogenesis of some autoimmune diseases, for example, primary Sjogren's syndrome (6) and juvenile dermatomyositis (7). Coculture experiments showed that Tfh2 and Tfh17, but not Tfh1, could efficiently induce immunoglobulin production from naive B cells by secreting interleukin 21 (IL-21) (8). And the number of Tfh cells is significantly decreased in PSS patients, suggesting that it is associated with pathogenesis (9, 10). Whereas, The role or function of Tfh cell subsets in PSS has not been reported in any studies.

This study determined the expressions of Tfh subsets Tfh1, Tfh2, and Tfh17 in PB of PSS patients in the active stage and remission after treatment, to analyze the pathogenic role of Tfh cells in PSS patients and its potential as a novel regulatory target.

## Materials and Methods

### Reagents and instruments

Fetal bovine serum (FBS), 1640 medium, Dulbecco's Modified Eagle Medium (DMEM) medium, phosphate buffer saline (PBS), trypsin, streptomycin, and penicillin were purchased from KeyGen Biotech (Nanjing, China); Ficoll separation medium and dimethyl sulfoxide (DMSO) from Bio-swamp company (Wuhan, China); CD4, CD45RA, CXCR5, CCR6, CXCR3 and corresponding isotype control antibodies from Biolegend Company, USA; EPICS XL flow cytometer from BECKMAN COULT ER Company.

### Sample collection

Ethylenediaminetetraacetic acid (EDTA) anticoagulation whole blood from 30 PSS patients, 30 PSS patients whose autoantibodies decreased significantly after treatment (as far as possible to trace the same patient), and 30 healthy controls of same sex and age were collected and stored in -70 °C refrigerator. This experiment strictly followed the Helsinki Declaration and obtained the informed

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consent signed by the patient. In addition, this experiment was approved by the Ethics Committee of Nanjing Gaochun People's Hospital (2021-162-01).

### Cell separation

Venous blood 3 mL of fasting man was collected in an EDTA-K2 vacuum anticoagulation blood collection tube (under sterile conditions). Peripheral blood mononuclear cells (PBMC) were isolated with Ficoll density gradient separation. To put it simply, take anticoagulant venous blood and fully mix it with the same amount of Hank's solution, and slowly add it to the stratified liquid surface along the tube wall with a dropper. Horizontal centrifugation at 2000 rpm for 20 min. Use capillaries to insert into the cloud layer and absorb mononuclear cells. Put another short medium tube, add 5 times of Hank's solution, centrifuge at 1500 rpm for 10 min, and wash the cells twice. Then, detected by a flow cytometer (Beckman Coulter EPICS XL flow cytometer). Diva software (BD Biosciences, version 1.1.3) was used to collect and analyze the data.

### Flow cytometry

The collected cells were rinsed twice with PBS, quantified, and then inoculated in each tube at a concentration of $1.0\times10^6$ cells. For the staining of cell surface markers, different fluorescently labeled antibodies (CD4, CD45RA, CXCR5, CCR6, CXCR3) and the corresponding isotype control antibodies were added into each tube and incubated for 30 min in dark at 4 °C. After rinsing the cells twice with PBS, resuspend the cells in 300 μL of PBS.

### Enzyme-linked immunosorbent assay

The PB was collected and centrifuged (2,000 r/min, 5 min). The supernatant was collected. The specific operations are as follows:

1) Added 50 μL of tissue fluid to be tested in each well of the micro-reaction plate, set up 2 wells for each positive and negative control, added 1 drop of positive control to each well, and set up 1 well of blank control.

2) Added 1 drop of enzyme conjugate to each well, mixed well, sealed the plate, and incubate at 37 °C for 30 min.

3) Discarded the liquid in the wells, filled the wells with washing liquid, let sit for 5 seconds, spin-dry, repeated 5 times, and patted dry.

4) Added 1 drop of each kind of color developer solution A and B to each well, mixed well, sealed the plate, and placed in a 37 °C incubator for 15 min.

5) Added 1 drop of stop solution to each well and mixed well.

6) Read the OD value of each well at the wavelength of 450 nm on a $V_{max}$ Kinetic Microplate Reader (Molecular Devices) after zero calibration for the blank well.

### Index detection

1) Collected PB from PSS and healthy recruiters, isolated PBMC, and detected Tfh subsets Tfh1, Tfh2, and Tfh17 by flow cytometry. To analyze the relationship between Tfh subsets (Tfh1, Tfh2, Tfh17) and SS disease activity index (SSDAI) in PB of PSS patients. SSDAI is an effective tool to measure disease activity (11, 12).

2) Collected PB from PSS patients in active and remission stages after treatment, respectively. Isolated PBMC, and detected the proportions of Tfh1, Tfh2, and Tfh17 by flow cytometry. The correlation between these proportions in the healthy group, primary group, active stage, and remission stage were analyzed.

3) IL-21 expressions in PSS patients in the active and remission stage were compared.

### Statistical methods

Data analysis was conducted by Graph Pad Prism 5 software (Graph Pad Prism, San Diego, CA, United States); cell counts were displayed as percentages, and data were expressed as mean ± standard deviation. Correlation analysis was adopted using Pearson's correlation. An paired T-test was employed to contrast different groups, and the boundary of statistical significance was set as $P<0.05$.

## Results

### Relationship between Tfh1, Tfh2, and Tfh17 levels and disease severity

Detected Tfh1, Tfh2, and Tfh17 in PSS patients by flow cytometry and analyzed their relationship with SSDAI. The results are shown in Figure 1-3: in vivo, Tfh1 level > Tfh17 level > Tfh2; Tfh2 and Tfh17 levels in $CD4^+$ T cells were notably decreased, and they exhibited a negative linear relationship with the severity of the disease increases.

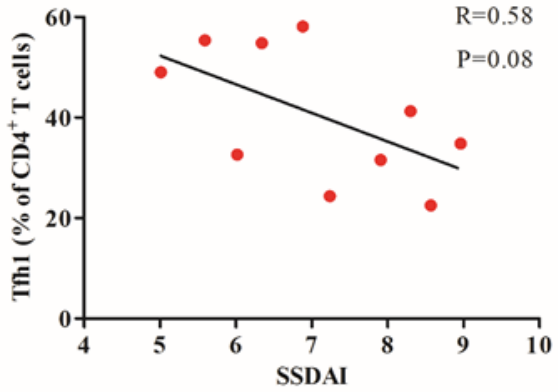


**Figure 1.** Relationship of Tfh1 proportion with SS disease activity index (SSDAI) in $CD4^+$ T cells.

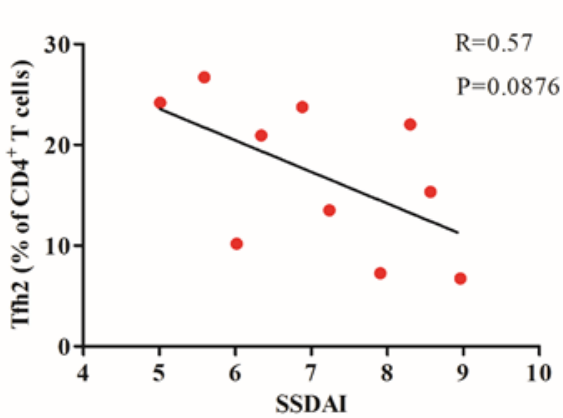


**Figure 2.** Relationship of Tfh2 proportion with SSDAI in $CD4^+$ T cells.

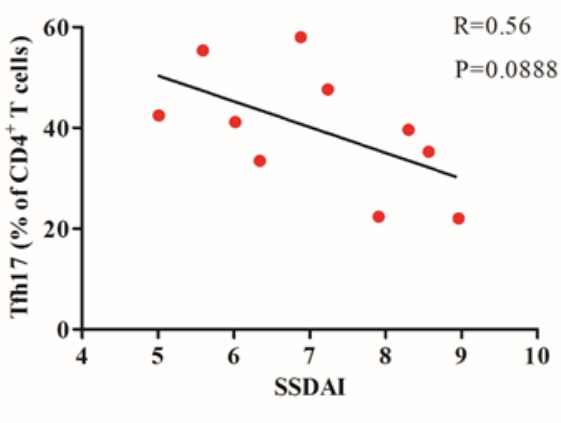


**Figure 3.** Relationship of Tfh17 proportion with SSDAI in $CD4^+$ T cells.

### Expression levels of Tfh1, Tfh2, and Tfh17 in different experimental groups

As Figure 4 shows, Tfh1 level > Tfh17 level > Tfh2 level in vivo; and Tfh1, Tfh2, and Tfh17 showed higher levels in the most healthy controls than in PSS patients and showed significantly higher levels in remission stage than in active stage. With the aggravation of the disease, these levels gradually decreased, while increasing during the remission period. This is corroborated by the results in Figures 1-3, indicating that the levels of Tfh1, Tfh2, and Tfh17 are negatively correlated with the severity of PSS.

### IL-21 level in SS patients in active and remission stages

As shown in Figure 5, IL-21 expression in SS patients in the active phase is significantly higher than that in the remission phase. This may be because the effector IL-21 of TFH cells participated in B cell proliferation and survival, promotes the transformation of B cells into plasma cells that can produce immunoglobulins, and thus plays a critical role in B cell-mediated SS.

### Discussion

Studies have shown that the pathogenesis of PSS is associated with changes in T lymphocyte subsets. The Tfh cells act as a vital role in humoral immunity, helping B cells mature and produce antibodies (13, 14). The Tfh cells are newly discovered as a subset of CD4+ helper T cells, directed by the chemokine receptor CXCR5 and localized to germinal centers to help B cells generate humoral immune responses. Recently, the importance of Tfh cell function in autoimmune diseases has gradually been recognized (15, 16).

Abnormal expression of Tfh-related molecules has been found in autoimmune diseases. Clinical studies have demonstrated that in rheumatoid arthritis (RA) patients, CD4 + CXCR5 + Foxp3 - Tfh cells increase, but CD4 + CXCR5 + Foxp3 + Tfr cells decrease, and the balance of Tfh/Tfr cells is disrupted. In the remission stage of RA patients, the Tfr cells increase and are negatively correlated with rheumatoid factor, which can inhibit the autoimmune response (17-19). It was reported that, compared with healthy people, patients with myasthenia gravis (MG) had increased Tfh cells, which was correlated with clinical severity, and the proportion of Tfr cells was decreased. After immunotherapy for MG patients, the patients' condition improved with their immunomodulatory factors and the recovery of the Tfr/Tfh cell ratio appeared (20). There are few studies concerning the relationship of PSS with Tfh cells. Therefore, we analyzed Tfh cell abnormalities in PSS patients in this study, which provided a certain theoretical foundation for clinical diagnosis and therapy.

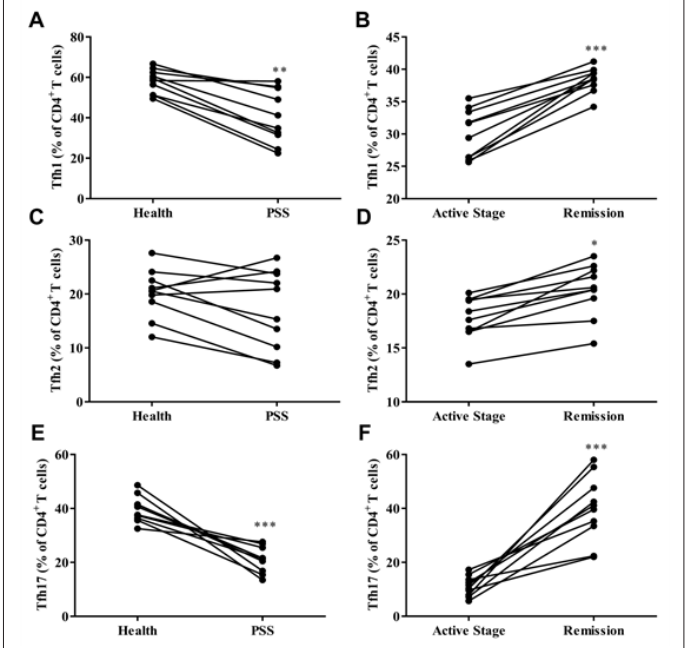


**Figure 4.** Expression levels of Tfh1, Tfh2, and Tfh17. A-F. Comparisons of Tfh1, Tfh2, and Tfh17 proportions between health and PSS groups, between active stage and remission groups. *$P$<0.05, **$P$<0.01 and ***$P$<0.001 *vs.* Health or Active Stage group.

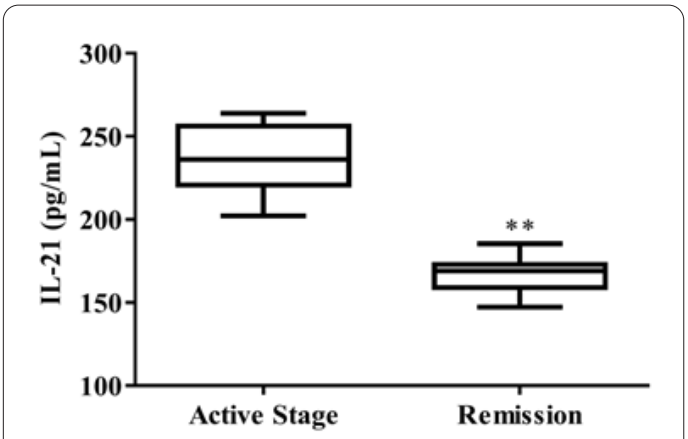


**Figure 5.** IL-21 expression in the active stage and remission stage in PSS patients. **$P$<0.01 *vs.* Active Stage group.

In this study, flow cytometry was applied to detect the proportions of Tfh1, Tfh2, and Tfh17 in PSS patients. The results showed that Tfh1 level > Tfh17 level > Tfh2 level. With the increase in disease severity, the content of Tfh1, Tfh2, and Tfh17 in $CD4^+$ T cells decreased significantly and showed a negative linear relationship with disease severity. See Figures 1-3 for details.

In order to further explore the mechanism, we also analyzed ratios of Tfh1, Tfh2, and Tfh17 in the remission stage after treatment. The results indicated that the levels of Tfh1, Tfh2, and Tfh17 were higher in most healthy controls than in PSS patients, and were remarkably lower in the active stage than that in the remission stage. Their contents gradually decreased with the aggravation of the

disease and increased in the remission stage. This is mutually supported by the results in Figures 1-3, indicating that the contents of Tfh1, Tfh2, and Tfh17 were negatively correlated with the severity of PSS.

The above findings showed that the proportion of Tfh in the PSS group was notably lower than that in the normal group, suggesting that Tfh T cell subsets had a close association with PSS pathogenesis. Nevertheless, However, this hypothesis has not been verified and needs further study. The pathogenesis of PSS has always been a hot spot for scholars at home and abroad. Some scholars have used immunohistochemical methods to detect the levels of Tfh cell markers CXCR5 and IL-21 in the labial gland of PSS patients. It has been found that CXCR5 and IL-21 levels in the labial gland of PSS patients were increased. It is believed that Tfh cells are related to the pathogenesis of PSS. The reason may be that IL-21, the main effector of Tfh cells, involves in the proliferation and survival of B cells and promotes the transformation of B cells into plasma cells that can produce immunoglobulins, thus playing a vital role in B cell-mediated autoimmune diseases and allergic diseases (21, 22). Studies have found that IL-21 expression increased in the lip gland tissue of PSS patients, which is positively correlated with cell apoptosis in the tissue, indicating that Tfh cells may participate in PSS pathogenesis by affecting cell apoptosis (23, 24).

In addition, foreign studies have found that IL-21 can induce B cell apoptosis, inhibit the growth of lymphoma cells and spawn its apoptosis. It is believed that IL-21 may promote the apoptosis of the lip gland tissue of PSS and then affect its pathogenesis. Furthermore, studies have found that Tfh cells can finally trigger the expression of apoptosis factor BCL-6, mediate B cell maturation, and produce various antibodies (25, 26).

To this end, we further examined IL-21 expression in the active and remission phases of PSS patients. The outcomes showed that the IL-21 level in the active phase was notably higher than that in the remission phase. This may be because the effector IL-21 of Tfh cells participated in B cell proliferation and survival, promoted the conversion of B cells to plasma cells that can produce immunoglobulin, and thus played a significant role in B cell-mediated SS.

In summary, the expression levels of tfh1, tfh2, and tfh17 were negatively correlated with the severity of PSS. The levels of IL-21 in PSS patients were significantly higher in the active phase than in the remission phase.

## Declarations

### Conflicts of interest
The authors state that there are no conflicts of interest.

### Ethics approval and consent to participate
The experimental protocol was approved by the Ethics Committee of Nanjing Gaochun People's Hospital (2021-162-01).

### Consent for publication
Not applicable.

### Availability of data and material
All data generated or analyzed during this study are included in this article.

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### Authors' contributions
WH conceived and designed the study. WH conducted most of the experiments. MM analyzed the data and performed the literature search and data extraction. WH drafted the manuscript. DK finalized the manuscript. All authors read and approved the final manuscript.

### Acknowledgments
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