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**Follicular helper T cells may play an important role in the severity of primary Sjögren's syndrome**

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**Abstract**

The aim of this study was to investigate the possible role of follicular helper T ( $T_{FH}$ ) cells in the pathogenesis of primary Sjögren's syndrome (pSS) by analyzing immune-competent cells and serological markers with special emphasis on clinical symptoms. We enrolled 50 pSS patients and 16 healthy individuals in the study. Patients had elevated ratio of peripheral  $T_{FH}$  cells, however, when dividing patients into two groups defined by the presence of extraglandular manifestations (EGMs), only patients with EGMs differed from controls significantly. Moreover,  $T_{FH}$  cell percentages correlated positively with both activated T cell and Tr1 cell values. On the contrary,  $T_{FH}$  cell percentages showed negative correlation with both IgM and IgG memory B cell proportions. Elevated  $T_{FH}$  percentages were observed in the anti-SSA/SSB positive patients, and also in patients with higher IL-12, IL-21 levels and focus score values. Increased  $T_{FH}$  cell proportions seem to have an important role in disease development.

**Keywords**

primary Sjögren's syndrome (pSS); follicular helper T cells ( $T_{FH}$ ); memory B cells; activated T cells; autoantibodies

**Abbreviations**

AID: activation-induced cytidine deaminase; APC: allophycocyanin; BAFF: B cell activating factor; Bcl-6: B cell lymphoma 6 protein; Blimp-1: B lymphocyte induced maturation protein 1; CD: Cluster of Differentiation; CCR7: C-C chemokine receptor type 7; C-X-C chemokine ligand 13: CXCL13; C-X-C chemokine receptor 5: CXCR5; DCs: dendritic cells; EGMs: extraglandular manifestations; ELISA: enzyme-linked immunosorbent assay; FITC: fluorescein isothiocyanate; FMO: Fluorescence Minus One; GC: germinal center; HLA: human leukocyte antigen; ICOS: inducible T cell co-stimulator; IFN: interferon; IL: interleukin; NK: natural killer; PD-1: programmed cell death protein 1; PE: R-phycoerythrin; PE-Cy5: R-phycoerythrin-Cyanine dye 5; PerCP-Cy5.5: Peridinin-chlorophyll protein-Cyanine dye 5.5; PI3K: Phosphatidylinositol 3-kinase; pSS: primary Sjögren's syndrome; RA: rheumatoid arthritis; SAP: signaling lymphocytic activation molecule (SLAM)-associated protein; SLE: systemic lupus erythematosus; STAT: signal transducer and activator of transcription protein;  $T_{FH}$ : T follicular helper; Tc: T cytotoxic; Th: T helper; Tr1: Type 1 regulatory T; Treg: regulatory T

## 1. Introduction

Primary Sjögren's syndrome (pSS) is a common systemic autoimmune disease, characterized by lymphocytic infiltration and destruction of the exocrine glands, primarily the lachrymal and salivary glands. Besides the characteristic glandular symptoms, other systemic symptoms, denoted as extraglandular manifestations (EGMs) such as non-erosive polyarthritis, vasculitis or myositis may also occur during the disease course [1].

Humoral autoimmune responses, B cell activation and autoantibody production are key immune abnormalities in pSS. Immunohistological analysis of biopsies from minor salivary glands usually demonstrates the presence of ectopic germinal centers (GCs) in the disease. The number of GCs in salivary glands correlates with the severity of inflammation, and enhanced anti-Ro/SSA, anti-La/SSB autoantibody-production. Moreover, the formation of ectopic GCs carries a higher risk of developing B cell lymphoma [2-4]. The selection of mutated high-affinity GC B cells depends on the restimulation with antigen arrayed on follicular dendritic cells and the provision of help by follicular helper T ( $T_{FH}$ ) cells [5].  $T_{FH}$  cells were initially proposed as a separate lineage, based on their failure to express T helper (Th) 1 /Th2/Th17 cytokines and lineage-specific transcription factors. Later investigations showed that  $T_{FH}$  cells may express characteristic cytokines of canonical helper T effector subsets, including interferon-gamma ( $IFN-\gamma$ ), interleukin-4 (IL-4) or IL-17 under specific microenvironment and accordingly,  $T_{FH}$  cells seem to be heterogeneous and have a close relationship to Th1, Th2 or Th17 cells [6-9]. A recent study demonstrated the ability of  $T_{FH}$  cells to form memory cells which differentiate upon recall not only into  $T_{FH}$  but conventional helper T cells as well [10]. Furthermore, two elegant studies showed that mouse Th2 and natural regulatory T cells (Treg) may transform into  $T_{FH}$  cells under certain in vitro conditions [8, 11]. These observations shed light on the developmental plasticity of  $T_{FH}$  cells. Therefore,

differing from other CD4<sup>+</sup> T cell lineages, T<sub>FH</sub> cells are mainly located in secondary lymphoid organs and defined by the expression of unique combination of cell surface molecules [9]. The main features of T<sub>FH</sub> cells arise from the expression of the B-cell lymphoma 6 (Bcl-6) transcription factor which regulates the expression of C-X-C chemokine receptor 5 (CXCR5), CXCR4 and C-C chemokine receptor type 7 (CCR7) that directs T<sub>FH</sub> cells into the C-X-C chemokine ligand 13 (CXCL13) rich areas of B cell follicles. Moreover it induces the expression of inducible T cell co-stimulator (ICOS), programmed cell death protein 1 (PD-1), cluster of differentiation (CD)40L and signaling lymphocytic activation molecule (SLAM)-associated protein (SAP) which are critical in T-B cell interaction [9, 12-16]. In the follicles, T<sub>FH</sub> cells provide survival signals to GC B cells via multiple pathways, including CD40L, IL-4, IL-21, PD-1, and B cell activating factor (BAFF), which compete with Fas-FasL interactions [14, 17, 18]. T<sub>FH</sub> cells are required for the formation and maintenance of GCs and for the generation of most memory B cells and long-lived plasma cells. The control of these processes hinges on T<sub>FH</sub> regulation of multiple B cell fate decisions, including cell death [14, 19]. IL-27 seems to be also important in T<sub>FH</sub> cell function and normal or pathogenic GC responses. In vivo IL-27 receptor is required on CD4<sup>+</sup> T cells for normal T<sub>FH</sub> cell generation, GC formation and antibody responses [20].

Similarly to pSS, ectopic lymphoid structures have also been described in the target tissues of other autoimmune conditions that are accompanied by B cell disturbances and autoantibody production, such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and autoimmune thyroiditis [21-23]. The better understanding of the B cell disturbances and development of ectopic GCs may provide new strategies for B cell targeted therapies. In the present study, in order to explore the possible role of T<sub>FH</sub> cells in the pathogenesis of pSS, we analyzed a wide spectrum of immune-competent cells and serological markers with a special emphasis on the clinical symptoms.

## 2. Material and methods

### 2.1. Patients

Fifty patients with pSS (2 male, 48 female) were enrolled in the study, recruited from the outpatient clinic for systemic autoimmune diseases at the Division of Clinical Immunology, Institute of Medicine, Medical and Health Science Center, University of Debrecen, where they received regular follow-up treatment. The diagnosis was based on the European-American consensus criteria. Sixteen age- and sex-matched healthy individuals served as controls [24].

Among pSS patients, 25 suffered from extraglandular manifestations (EGMs), while 25 had only glandular symptoms. The distribution of EGMs of pSS patients was as follows: polyarthritis n=19, Raynaud's phenomenon n=11, lymphadenopathia n=3, vasculitis n=3, polyneuropathia n=2 and myositis n=1. No patients, or controls enrolled in this study took any immunosuppressive or immunomodulating medications, or had ongoing or previous infections during the study. Informed written consent was obtained from the subjects, and the study has been approved by the Ethics Committee of the University of Debrecen. All experiments carried out were in compliance with the Declaration of Helsinki. Data on subjects enrolled in the study are summarized in Table 1.

### 2.2. Determination of lymphocyte subpopulations

For phenotypic analysis from heparinized blood samples we used CD3-fluorescein isothiocyanate (FITC), CD4-FITC, CD8-R-phycoerythrin (PE), CD19-R-phycoerythrin-Cyanine dye 5 (PE-Cy5), and CD16+CD56-PE (BD Biosciences, San Diego, CA, USA and

Immunotech, Beckmann Coulter Company, Marseille, France) monoclonal antibodies against cell surface markers. The expression of T-lymphocyte activation markers such as CD69-PE-Cy5 and human leukocyte antigen (HLA)-DR-PE were also determined on CD3<sup>+</sup> cells (BD Biosciences). The following monoclonal antibody combinations were used for phenotypic characterization of naive and memory T cells: CD45RA-FITC/CD4-PE/CD62L-PE-Cy5 (Immunotech) and CD45RA-FITC/CD8-PE/CD62L-PE-Cy5 (Serotec, Oxford, UK and Immunotech). For identification naive and memory B cells we used IgD-FITC/CD27-PE/CD19-PE-Cy5 (Beckman Coulter Inc, Fullerton, CA, USA and Immunotech). We also investigated CD4<sup>+</sup>CD25<sup>bright</sup> Treg cells with the following reagents: CD4-FITC (Sigma Aldrich, St. Louis, MO, USA), CD25-PE-Cy5 (Immunotech). Samples were processed according to the Coulter Q-PREP protocol and system (Beckman Coulter Inc, Miami, FL, USA), as described previously [25, 26]. Measurements were performed on a Coulter FC500 flow cytometer (Beckman Coulter Inc.). Isotype-matched antibodies were used in all procedures. The following peripheral immune-competent cell types were investigated: T cells (CD3<sup>+</sup>), T-helper cells (CD4<sup>+</sup>), cytotoxic T (Tc) cells (CD8<sup>+</sup>), B cells (CD19<sup>+</sup>), early-activated T lymphocytes (CD3<sup>+</sup>CD69<sup>+</sup>), late-activated T lymphocytes (CD3<sup>+</sup>HLADR<sup>+</sup>), natural killer (NK) cells (CD3<sup>-</sup>CD56<sup>+</sup>CD16<sup>+</sup>), NKT cells (CD3<sup>+</sup>CD56<sup>+</sup>CD16<sup>+</sup>) and CD4<sup>+</sup>CD25<sup>bright</sup> Treg cells (CD4<sup>+</sup>CD25<sup>bright</sup>). The B, T, T-helper, activated T, NK, NKT and Treg cells were quantified as their percentage in the entire lymphocyte population. Naive and memory T cell subsets were determined as their percentages in CD4<sup>+</sup> or CD8<sup>+</sup> cells, as the followings: naive helper T (CD4<sup>+</sup>CD45RA<sup>+</sup>CD62L<sup>+</sup>), central memory helper T (CD4<sup>+</sup>CD45RA<sup>-</sup>CD62L<sup>+</sup>), effector memory helper T (CD4<sup>+</sup>CD45RA<sup>-</sup>CD62L<sup>-</sup>), naive cytotoxic T (CD8<sup>+</sup>CD45RA<sup>+</sup>CD62L<sup>+</sup>), central memory cytotoxic T (CD8<sup>+</sup>CD45RA<sup>-</sup>CD62L<sup>+</sup>), effector memory cytotoxic T (CD8<sup>+</sup>CD45RA<sup>-</sup>CD62L<sup>-</sup>) and terminally differentiated effector memory cytotoxic T cells (CD8<sup>+</sup>CD45RA<sup>+</sup>CD62L<sup>-</sup>). Naive and



memory B cell values were calculated as their percentages in CD19<sup>+</sup> B cells as the followings: naive B (IgD<sup>+</sup>CD27<sup>-</sup>), IgM memory B (IgD<sup>+</sup>CD27<sup>+</sup>), IgG memory B cells (IgD<sup>-</sup>CD27<sup>+</sup>).

For the identification of CD4<sup>+</sup> T helper cell subsets we used intracytoplasmic cytokine staining method which has been described previously [25, 26]. The following combinations of monoclonal antibodies were used: IFN- $\gamma$ -FITC/IL-4-PE/CD4-PE-Cy5 or CD8 PE-Cy5, IL-10-PE/CD4-PE-Cy5 or CD8 PE-Cy5 (all from BD Biosciences), IL-17-PE/IFN- $\gamma$ -FITC/CD4-PE-Cy5 (R&D Systems, Minneapolis, MN, USA and BD Biosciences). Measurements were performed and data were collected on a Coulter FC500 flow cytometer (Beckman Coulter Inc.). Isotype-matched antibodies were used in all procedures. The phenotypes within CD4<sup>+</sup> cells were determined as follows: Th1 cells (CD4<sup>+</sup> IFN- $\gamma$ <sup>+</sup> IL-4<sup>-</sup>); Th2 cells (CD4<sup>+</sup> IFN- $\gamma$ <sup>-</sup> IL-4<sup>+</sup>); Th17 cells (CD4<sup>+</sup> IFN- $\gamma$ <sup>-</sup> IL17<sup>+</sup>) and Type 1 regulatory T cells (Tr1) (CD4<sup>+</sup> IL-10<sup>+</sup>). Cells were quantified as their percentage in the CD4<sup>+</sup> lymphocyte population.

### **2.3. Assessment of T<sub>FH</sub> cells in the peripheral blood**

For the identification of circulating T<sub>FH</sub> cells we used CD4-allophycocyanin (APC), CXCR5-Alexa Fluor 488, ICOS-PE and PD-1-Peridinin-chlorophyll protein-Cyanine dye 5.5 (PerCP-Cy5.5) (BD Biosciences) monoclonal antibodies against human cell surface molecules. Fluorescence Minus One (FMO) controls were used in all procedures. FMO controls contain every stain in the panel except the one we are controlling, therefore this method are ideal for determining positive vs. negative population in the sample. The stained cells were assessed using the FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and data analysis was performed using FlowJo Software (Treestar, Ashland, OR, USA). At least 35 000 events per sample were analyzed within the lymphocyte population. T<sub>FH</sub> cells were quantified as their percentage in the CD4<sup>+</sup> lymphocyte population.

#### **2.4. Evaluation of serum soluble cytokines**

Circulating cytokine levels including IL-12, IL-21 and IL-27 were determined by Platinum ELISA Kits (all from eBioscience, San Diego, CA, USA) and were used according to the manufacturer's instructions. Absorbance measurements were detected with a LabSystems Multiskan MS Microplate Reader (Labsystems, Helsinki, Finland) at 450 nm, data were analyzed by Genesis 2.0 software (Labsystems).

#### **2.5. Determination of anti-SSA and anti-SSB**

Anti-Ro/SSA and anti-La/SSB autoantibodies were determined by ELISA technique with AUTOSTAT II kits (Hycor Biomedical, Indianapolis, IN, USA) according to the manufacturer's instructions.

#### **2.6. Statistical Analysis**

Data were represented and statistical analyzed with GraphPad Prism 5 software (Graphpad Software, San Diego, USA). We determined the extreme outlier scores by boxplot using SPSS version 16.0 (SPSS, Inc., IL, USA), then excluded them from the data. To assess the distribution of the data Kolmogorov–Smirnov and Shapiro-Wilk normality test were used. In cases of normal distribution, if the F probe was granted we used unpaired two-sample t test, otherwise it was not granted we used Welch's t test for statistical comparison of the experimental data. In cases if distributions the data set was different from normal, the Mann–Whitney U test was used. This statistical method has the assumption of homogeneity of variances which was tested using Levene's test in SPSS. The correlations between two

variables were evaluated with Spearman's rank correlation coefficient. Differences were considered statistically significant at  $p < 0.05$ .

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### 3. RESULTS

#### 3.1. $T_{FH}$ cells in peripheral blood of pSS patients and healthy individuals

In our research, first we gated on lymphocytes according to forward and side scatter, then we gated on  $CD4^+CXCR5^+$  T cells. Within the  $CD4^+CXCR5^+$  lymphocytes we determined the fraction of  $ICOS^+PD-1^+$  T cells from peripheral blood in patients with pSS and healthy controls by flow cytometry (Fig. 1A-C). According to our results, the percentages of  $CD4^+CXCR5^+ICOS^+$  T cells were significantly elevated in patients with EGMs when compared to patients with glandular symptoms and healthy controls (mean percentages of pSS with EGMs vs. controls:  $1.40\% \pm 0.68$  vs  $0.52\% \pm 0.32$ , respectively,  $p < 0.0001$  and pSS with EGMs vs. pSS without EGMs:  $1.40\% \pm 0.68$  vs  $0.47\% \pm 0.26$ , respectively,  $p < 0.0001$ ) (Fig. 1D). The percentages of  $CD4^+CXCR5^+PD-1^+$  T cells were also significantly elevated in patients with EGMs when compared to patients without EGMs and healthy controls (median percentages of pSS with EGMs vs. controls:  $4.67$  (2.15-7.54) % vs.  $2.98$  (1.88-9.25) %, respectively,  $p = 0.0039$  and pSS with EGMs vs. pSS without EGMs:  $4.67$  (2.15-7.54) % vs.  $3.33$  (2.14-7.42) %, respectively,  $p = 0.0102$ ) (Fig. 1E). However, there were no significant differences between patients with glandular symptoms and the control group (in case of  $CD4^+CXCR5^+ICOS^+$  T cells, the mean percentages of pSS without EGMs vs. controls:  $0.47\% \pm 0.26$  vs  $0.52\% \pm 0.32$ , respectively,  $p = 0.5795$  while  $CD4^+CXCR5^+PD-1^+$  T cells, the median percentages of pSS without EGMs vs. controls:  $3.33$  (2.14-7.42) % vs  $2.98$  (1.88-9.25) %, respectively,  $p = 0.2140$ ) (Fig. 1D and E). Among  $CD4^+CXCR5^+$  T cells, we compared the percentages of  $ICOS^+$  and  $PD-1^+$  cells and a moderate correlation has been found between the two groups ( $R = 0.4640$ , respectively,  $p = 0.0008$ ) (Fig. 1F). Next we examined these two surface molecules simultaneously on the cells and we found significant

higher percentages of T<sub>FH</sub> cells in patients with EGM than in patients with glandular symptoms only and healthy controls (mean percentages of pSS with EGMs vs. controls:  $0.79\% \pm 0.39$  vs  $0.29\% \pm 0.19$ , respectively,  $p < 0.0001$  and pSS with EGMs vs. pSS without EGMs:  $0.79\% \pm 0.39$  vs  $0.26\% \pm 0.13$ , respectively,  $p < 0.0001$ ) (Fig. 1G). The proportions of T<sub>FH</sub> cells in patients without EGMs did not differ from control values (mean percentages of pSS without EGMs vs. controls:  $0.26\% \pm 0.13$  vs  $0.29\% \pm 0.19$ , respectively,  $p = 0.5555$ ) (Fig. 1G).

### **3.2. Correlation analysis between peripheral T<sub>FH</sub> cells and lymphocyte populations in patients with pSS**

We analyzed the possible associations between the percentages of T<sub>FH</sub> cells and other investigated lymphocyte populations. We found positive correlations between the proportions of early-activated T cells and T<sub>FH</sub> cells ( $R = 0.4147$ , respectively,  $p = 0.0028$ ) (Fig. 2A), the percentages of late-activated T cells and T<sub>FH</sub> cells ( $R = 0.3148$ , respectively,  $p = 0.0276$ ) (Fig. 2B) as well as the proportions of Tr1 cells and T<sub>FH</sub> cells ( $R = 0.4140$ , respectively,  $p = 0.0034$ ) (Fig. 2C). A significant negative correlation was observed between the percentages of IgM memory B cells and T<sub>FH</sub> cells ( $R = -0.3104$ , respectively,  $p = 0.0300$ ) (Fig. 2E) as well as between the percentages of IgG memory B cells and T<sub>FH</sub> cells ( $R = -0.3111$ , respectively,  $p = 0.0314$ ) (Fig. 2F). There was no significant correlation between T<sub>FH</sub> cells and the other investigated lymphocyte subpopulations.

### 3.3. Correlation analysis between antibodies and T<sub>FH</sub> cells in patients with pSS

Among pSS patients, we found 19 anti-Ro/SSA positive individuals, 11 of them were anti-Ro/SSA – anti-La/SSB double positive. We evaluated the associations between the presence of antibodies and peripheral T<sub>FH</sub> cells. We observed a significant positive correlation between the levels of serum IgG antibody and the percentages of peripheral T<sub>FH</sub> cells ( $R = 0.2971$ , respectively,  $p = 0.0403$ ) (Fig.2D). Additionally, we investigated the association of autoantibody titers with the proportions of T<sub>FH</sub> cells in pSS patients. We found significant positive correlation between the levels of anti-Ro/SSA antibody and the percentages of T<sub>FH</sub> cells ( $R = 0.5143$ , respectively,  $p = 0.0243$ ) (Fig.2G), as well as between the levels of anti-La/SSB antibody and the proportions of T<sub>FH</sub> cells ( $R = 0.6545$ , respectively,  $p = 0.0336$ ) (Fig. 2H) in the antibody-positive group. Interestingly, when we analyzed the percentages of T<sub>FH</sub> cells in patients with pSS with emphasis on the presence of autoantibodies, the percentages of T<sub>FH</sub> cells showed a significant 2-fold increase in anti-Ro/SSA antibody-positive group compared to healthy controls (median percentages of T<sub>FH</sub> cells 0.510 (0.070-1.560) % vs. 0.240 (0.060-0.750) %, respectively,  $p = 0.0009$ ) (Fig.2I) and approximately one and a half-fold increase compared to antibody-negative group (median percentages of T<sub>FH</sub> cells 0.510 (0.070-1.560) % vs. 0.350 (0.030-1.690) %, respectively,  $p = 0.0341$ ) (Fig. 4C). Regarding anti-La/SSB, we found significant 3.6-fold higher percentages of T<sub>FH</sub> cells in anti-La/SSB antibody-positive group compared to healthy controls (median percentages of T<sub>FH</sub> cells 0.870 (0.070-1.560) % vs 0.240 (0.060-0.750) %, respectively,  $p = 0.0052$ ) (Fig. 2I) and roughly 2.5-fold higher percentages, compared to antibody-negative group (median percentages of T<sub>FH</sub> cells 0.870 (0.070-1.560) % vs 0.350 (0.030-1.690) %, respectively,  $p = 0.0452$ ) (Fig. 2I). The anti-Ro/SSA and anti-La/SSB double antibody-positive group correspond to the anti-La/SSB

antibody-positive group, because patients who had anti-La/SSB antibody were positive for anti-Ro/SSA as well.

### 3.4. Increased levels of serum soluble cytokines in patients with pSS

We also examined the relationship between the concentrations of serum IL-12 and the percentages of T<sub>FH</sub> cells. Based on the titers of IL-12, we divided patients into two groups: (1) IL-12 negative group - the absorbances were lower than the dilution medium and (2) IL-12-positive group - the absorbances were higher than the dilution medium. The percentages of T<sub>FH</sub> cells showed a significant 3-fold increase in IL-12-positive group compared to healthy controls (median percentages of T<sub>FH</sub> cells 0.69 (0.24-1.56) % vs. 0.24 (0.06-0.75) %, respectively,  $p = 0.0011$ ) (Fig. 3A) and a significant 2-fold increase between the positive and negative groups (median percentages of T<sub>FH</sub> cells 0.69 (0.24-1.56) % vs. 0.35 (0.03-1.69) %, respectively,  $p = 0.0249$ ) (Fig. 3A). Similarly to the assessment of cytokine IL-12, for the evaluation of IL-21 levels we divided the patients into two groups based on the levels of serum IL-21. The percentages of T<sub>FH</sub> cells showed a significant 3,5-fold increase in IL-21-positive group compared to healthy controls (median percentages of T<sub>FH</sub> cells 0.87 (0.07-1.56) % vs. 0.24 (0.06-0.75) %, respectively,  $p = 0.0017$ ) (Fig. 3B) and a significant 2-fold increase between the IL-21-positive and IL-21-negative groups (median percentages of T<sub>FH</sub> cells 0.87 (0.07-1.56) % vs. 0.40 (0.03-1.69) %, respectively,  $p = 0.0186$ ) (Fig. 3B). Furthermore, based on the presence or absence of EGMs, we divided the IL-21 negative and positive groups into two subgroups. Concerning patients with higher levels of IL-21 cytokine, we found strong connection between the percentages of T<sub>FH</sub> cells and the presence of EGMs. The percentages of T<sub>FH</sub> cells showed a significant 1,6-fold increase in the patients with EGMs in IL-21-negative group compared to the patients with EGMs in IL-21-positive group (median

percentages of T<sub>FH</sub> cells 0.560 (0.130-1.690) % vs. 0.905 (0.510-1.560) %, respectively,  $p = 0.0414$ ) (Fig.3C). Whereas, no significant differences was observed between the patients with glandular symptoms in IL-21-negative group and the patients with glandular symptoms in IL-21-positive group (median percentages of T<sub>FH</sub> cells 0.235 (0.030-0.600) % vs. 0.310 (0.070-0.390) %, respectively,  $p = 0.9666$ ) (Fig.3C). We found no significant difference in the serum levels of IL-27 between pSS patients and healthy individuals.

### **3.5. The intensity of focus scores in association with T<sub>FH</sub> cells**

The histological findings were also examined in labial salivary gland biopsies of 14 pSS patients. Within the group of patients who suffering only glandular symptoms the distribution of focus scores was as follows: focus score of 1 ( $n = 2$ ) and focus score of 2 ( $n = 4$ ). In the group of patients with EGMs the distribution of focus scores was as follows: focus score of 2 ( $n = 2$ ), focus score of 3 ( $n = 3$ ) and focus score of 4 ( $n = 3$ ). We also examined the relationship between the percentages of T<sub>FH</sub> cells and biopsy focus scores and we found a significant positive correlation ( $R = 0.6984$ , respectively,  $p = 0.0055$ ) (Fig. 4).



#### 4. Discussion

Cellular and humoral mechanisms behind the immune disturbances characteristic to Sjögren's syndrome are still not known in details, but evidence suggests that both T and B cells infiltrating the exocrine glands play an important role in the disease development. A complex interplay between lymphocytes, macrophages, dendritic cells (DCs) and both activated epithelial and endothelial cells leads to autoimmune tissue damage and contributes to the disease progression [3, 27-29]. Systemic features of pSS, such as circulating immune complexes, hypergammaglobulinemia, organ-specific and non organ-specific autoantibodies, furthermore, ectopic GCs in the affected tissues and enhanced risk of developing B cell lymphoma underline the crucial role of B cells in the disease. The most intensively studied T helper cell type in recent years is the  $T_{FH}$  cell, which has a major role in the proliferation and differentiation into memory or plasma cells of antigen-specific B cells, and in some cases even contributes to the triggering of their apoptosis. Recent studies demonstrated elevated peripheral  $T_{FH}$  cell percentages in certain autoimmune conditions, such as RA, SLE and autoimmune thyroiditis [30-32].

In our present study, we assessed the frequency of  $T_{FH}$  cells in pSS patients by determining  $CD4^+CXCR5^+ICOS^+PD-1^+$  T cells. After analysing the results of the whole patient population, we found an elevated ratio of peripheral  $T_{FH}$  cells, interestingly, when we divided patients into two groups based on the presence or absence of EGMs, only patients with EGMs showed significant differences, while values of patients without EGMs were similar to healthy controls. Of note, a recent study focusing on SLE, has also investigated a small group of patients with Sjögren's syndrome. However, authors only identified peripheral  $CD4^+CXCR5^+ICOS^{high}$  and  $CD4^+PD-1^{high}$  cells, not  $CD4^+CXCR5^+ICOS^+PD-1^+$   $T_{FH}$  cells, moreover, laboratory values were not evaluated with the emphasis on clinical symptoms [30].

A recent study demonstrated that the proportions of CD4<sup>+</sup> CXCR5<sup>+</sup> CCR6<sup>+</sup> T cells were elevated in pSS patients and correlated with the disease activity. The workgroup's results also showed that these cells expressed the key features of T<sub>FH</sub> cells, including PD-1, ICOS, CD40L, IL-21 and Bcl-6 [33]. Our results also indicate that proportions of T<sub>FH</sub> cells in pSS patients show elevated values. Moreover, our study proved that the ratio of T<sub>FH</sub> cells is closely connected to the presence of systemic clinical symptoms in the patients, consequently, T<sub>FH</sub> cells may play an important role not only in the development of pSS, but also in the disease progression. Regarding other T cell subsets, our workgroup demonstrated previously that proportions of early activated T and Tr1 cells are elevated in the peripheral blood of pSS patients [25, 26]. In our present study, we found positive correlations between the percentages of T<sub>FH</sub> cells and early- and late-activated T cells, which indicate that in parallel with the activation of immune system, a stronger T<sub>FH</sub> cell expansion can be observed. T<sub>FH</sub> cells also showed a positive correlation with Tr1 cell percentages. Elevation in Tr1 cell proportions could be a part of an increased counter-regulatory reaction, presumably compensating the derailed, disproportional immune responses.

B cell hyperreactivity, development of autoantibodies, and disturbance in distribution of B cell subtypes on the periphery are characteristics in pSS. Levels of circulating IgM and IgG memory B cells are decreased in peripheral blood; on the contrary their proportions are elevated in the inflamed tissues, especially in salivary glands [2, 34-37]. Our results support partially the aforementioned observations, since we found a negative correlation between the proportions of T<sub>FH</sub> cells and IgM and IgG memory B cells in the peripheral blood. We were also interested in the associations between the percentages of T<sub>FH</sub> cells and the levels of IgG and autoantibodies. We found positive correlation between T<sub>FH</sub> cell values and IgG levels, which may be the result of the consequential B cell activation. Additionally, we observed elevated T<sub>FH</sub> percentages in the anti-Ro/SSA and anti-La/SSB positive patients, compared to

autoantibody negative patients and healthy controls. Moreover, the  $T_{FH}$  proportions showed positive correlation with anti-Ro/SSA and anti-La/SSB titers. Taken together, these observations are in line with the concept that the elevated  $T_{FH}$  profile plays an important role in autoantibody production.

Recent studies suggest that differentiation of  $T_{FH}$  cells can be supported by an integrated model, which consists of activation of cells, upholding of the activated condition and an entirely polarised state. Supposing that myeloid DCs are the only cell types capable of stimulating naive T cells, we can infer that myeloid DCs found in secondary lymphoid tissue, producing IL-12 cytokines play a central role in increasing amounts of  $T_{FH}$  cells in certain autoimmune diseases. A former study showed that activated myeloid DCs can induce the differentiation of  $CD4^+$  naive T cells into  $T_{FH}$  cells through the signal transducer and activator of transcription protein 4 (STAT4) pathway via IL-12 cytokine secretion. Furthermore, it has been demonstrated that IL-12 is capable of inducing IL-21, CXCR5, ICOS and Bcl-6 expression of human  $CD4^+$  naive T cells in vitro, thus promoting B cell antibody production, although the capacity of IL-12 to induce IL-21 cytokine expression is mostly STAT3 dependent [38-41]. Therefore we studied levels of soluble IL-12 cytokine in sera of pSS patients. Not every patient showed measurable levels of IL-12, thus we arranged them in two groups based on serum IL-12 levels: IL-12 positive and negative groups. According to our results, IL-12 positive patients had significantly higher percentages of  $T_{FH}$  cells. Another important signal molecule for  $T_{FH}$  cells is IL-21, which can contribute to extended survival of  $T_{FH}$  cells in an autocrine manner, via the activation of phosphatidylinositol 3-kinase (PI3K). Kroenke et al. recently reported that c-Maf, and not Bcl-6, induces the production of IL-21, nevertheless, both transcription factors work together to trigger the development of  $T_{FH}$  characteristics [13]. Moreover, co-stimulation controlled by IL-21 is necessary for high level expression of CXCR5, which is required for migration into GCs [42, 43]. Another important

role of IL-21 cytokine is to promote differentiation of B cells into plasma cells through the STAT3 pathway with inducing the B lymphocyte induced maturation protein 1 (Blimp-1) transcription factor [14]. Serum levels of IL-21 cytokine were found to be elevated in autoimmune thyroiditis and RA patients [31, 32]. In our study, not every patient had measurable levels of IL-21, thus we divided them into two groups based on IL-21 level in the serum: IL-21 positive and negative groups. We found that patients with higher T<sub>FH</sub> cell percentages had elevated IL-21 levels, moreover, these cytokine concentrations correlated with the presence of EGMs, thus supporting the theory, that IL-21 has an important role in immune processes regulated by T<sub>FH</sub> cells.

Additionally, our study revealed a close relationship between peripheral T<sub>FH</sub> cell percentages and the focus scores of labial salivary gland biopsies. Of note, patients with EGMs had higher focus scores compared to patients with only sicca symptoms. This interesting observation suggests that the elevated frequency of circulating T<sub>FH</sub> cells may be associated with the severity of glandular involvement.

## 5. Conclusions

Percentages of peripheral T<sub>FH</sub> cells are increased in pSS patients suffering from a more pronounced course of disease. Moreover, our results revealed clear correlations between elevated T<sub>FH</sub> cell percentages, certain B cell subtype proportions and autoantibody levels. Taken together, our observations raise the possibility that alteration in T<sub>FH</sub> cell proportions may play an important role in the disease development. Therefore, modulation of T<sub>FH</sub> cells could be a potentially powerful element of the novel therapeutic selection in pSS, by blocking T<sub>FH</sub> differentiation and their interaction with B cells using selective agents. We believe that further investigation of the complex function of T<sub>FH</sub> cells will open new avenues to understand the derailed B cell operation and autoimmune processes in pSS.

**Competing interests**

The authors declare that they have no competing interests.

**Authors' contributions**

KSz and GP participated in the study design, performed laboratory experiments, collected, statistically analyzed and interpreted the data, and drafted the manuscript. SB and EGy participated in the laboratory experiments. ASz participated in the interpretation of clinical data. MZ designed the study, interpreted the data and gave final approval of the version to be published. All authors read and approved the final manuscript.

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**References**

- [1] M. Zeher, Sjögren's syndrome., in: M. Zeher, P. Szodoray (Eds.) Sjögren's syndrome and associated disorders., Transworld Research Network, Kerala, India, 2009, pp. 1-25.
- [2] J. Bohnhorst, M. Bjørgan, J. Thoen, R. Jonsson, J. Natvig, K. Thompson, Abnormal B cell differentiation in primary Sjögren's syndrome results in a depressed percentage of circulating memory B cells and elevated levels of soluble CD27 that correlate with Serum IgG concentration., *Clin Immunol.*, 103 (2002) 79-88.
- [3] A. Hansen, P. Lipsky, T. Dörner, B cells in Sjögren's syndrome: indications for disturbed selection and differentiation in ectopic lymphoid tissue., *Arthritis Res Ther.*, 9 (2007) 218.
- [4] A. Illes, L. Varoczy, G. Papp, P. Wilson, P. Alex, R. Jonsson, T. Kovacs, Y. Kontinen, M. Zeher, B. Nakken, P. Szodoray, Aspects of B-cell non-Hodgkin's lymphoma development: a transition from immune-reactivity to malignancy., *Scand J Immunol.*, 69 (2009) 387-400.
- [5] C. Vinuesa, M. Linterman, C. Goodnow, K. Randall, T cells and follicular dendritic cells in germinal center B-cell formation and selection., *Immunol Rev.*, 237 (2010) 72-89.
- [6] R. Nurieva, Y. Chung, D. Hwang, X. Yang, H. Kang, L. Ma, Y. Wang, S. Watowich, A. Jetten, Q. Tian, C. Dong, Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. , *Immunity*, 29 (2008) 138–149.
- [7] I. King, M. Mohrs, IL-4-producing CD4+ T cells in reactive lymph nodes during helminth infection are T follicular helper cells., *J Exp Med*, 206 (2009) 1001–1007.
- [8] A. Zaretsky, J. Taylor, I. King, F. Marshall, M. Mohrs, E. Pearce, T follicular helper cells differentiate from Th2 cells in response to helminth antigens., *J Exp Med*, 206 (2009) 991–999.
- [9] M. Chen, Z. Guo, W. Ju, B. Ryffel, X. He, S. Zheng, The development and function of follicular helper T cells in immune responses., *Cell Mol Immunol.*, 9 (2012) 375-379.

- [10] K. Lüthje, A. Kallies, Y. Shimohakamada, G. Belz, A. Light, D. Tarlinton, S. Nutt, The development and fate of follicular helper T cells defined by an IL-21 reporter mouse., *Nat Immunol.*, 13 (2012) 491-498.
- [11] M. Tsuji, N. Komatsu, S. Kawamoto, K. Suzuki, O. Kanagawa, T. Honjo, S. Hori, S. Fagarasan, Preferential generation of follicular B helper T cells from Foxp3+ T cells in gut Peyer's patches., *Science.*, 323 (2009) 1488-1492.
- [12] C. Ma, E. Deenick, M. Batten, S. Tangye, The origins, function, and regulation of T follicular helper cells., *J Exp Med.* , 209 (2012) 1241-1253.
- [13] M. Kroenke, D. Eto, M. Locci, M. Cho, T. Davidson, E. Haddad, S. Crotty, Bcl6 and Maf cooperate to instruct human follicular helper CD4 T cell differentiation., *J Immunol.* , 188 (2012) 3734-3744.
- [14] S. Crotty, Follicular Helper CD4 T Cells (TFH). *Annu Rev Immunol*, 29 (2011) 621-663.
- [15] E. Deenick, C. Ma, The regulation and role of T follicular helper cells in immunity., *Immunology*, 134 (2011) 361-367.
- [16] E. Deenick, C. Ma, R. Brink, S. Tangye, Regulation of T follicular helper cell formation and function by antigen presenting cells., *Curr Opin Immunol*, 23 (2011) 111-118.
- [17] R. Spolski, W. Leonard, IL-21 and T follicular helper cells., *Int Immunol*, 22 (2010) 7-12.
- [18] M. Linterman, R. Rigby, R. Wong, D. Yu, R. Brink, J. Cannons, P. Schwartzberg, M. Cook, G. Walters, C. Vinuesa, Follicular helper T cells are required for systemic autoimmunity., *J Exp Med*, 206 (2009) 561-576.
- [19] M. Linterman, C. Vinuesa, Signals that influence T follicular helper cell differentiation and function., *Semin Immunopathol.*, 32 (2010) 183-196.
- [20] M. Batten, N. Ramamoorthi, N. Kljavin, C. Ma, J. Cox, H. Dengler, D. Danilenko, P. Caplazi, M. Wong, D. Fulcher, M. Cook, C. King, S. Tangye, F. de Sauvage, N. Ghilardi, IL-



27 supports germinal center function by enhancing IL-21 production and the function of T follicular helper cells., *J Exp Med*, 207 (2010) 2895-2906.

[21] A. Schröder, A. Greiner, C. Seyfert, C. Berek, Differentiation of B cells in the nonlymphoid tissue of the synovial membrane of patients with rheumatoid arthritis., *Proc Natl Acad Sci U S A*, 93 (1996) 221-225.

[22] A. Hutloff, K. Büchner, K. Reiter, H. Baelde, M. Odendahl, A. Jacobi, T. Dörner, R. Kroczeck, Involvement of inducible costimulator in the exaggerated memory B cell and plasma cell generation in systemic lupus erythematosus., *Arthritis Rheum.*, 50 (2004) 3211-3220.

[23] E. Hsi, T. Singleton, S. Svoboda, B. Schnitzer, C. Ross, Characterization of the lymphoid infiltrate in Hashimoto thyroiditis by immunohistochemistry and polymerase chain reaction for immunoglobulin heavy chain gene rearrangement., *Am J Clin Pathol.*, 110 (1998) 327-333.

[24] C. Vitali, S. Bombardieri, R. Jonsson, H. Moutsopoulos, E. Alexander, S. Carsons, T. Daniels, P. Fox, R. Fox, S. Kassan, S. Pillemer, N. Talal, M. Weisman, E.S.G.o.C.C.f.S.s. Syndrome., Classification criteria for Sjögren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group., *Ann Rheum Dis.*, 6 (2002) 554-558.

[25] P. Szodoray, G. Papp, I. Horvath, S. Barath, S. Sipka, B. Nakken, M. Zeher, Cells with regulatory function of the innate and adaptive immune system in primary Sjögren's syndrome., *Clin Exp Immunol.*, 157 (2009) 343-349.

[26] P. Szodoray, I. Gal, S. Barath, M. Aleksza, I. Horvath, P. Gergely, G. Szegedi, B. Nakken, M. Zeher, Immunological alterations in newly diagnosed primary Sjögren's syndrome characterized by skewed peripheral T-cell subsets and inflammatory cytokines., *Scand J Rheumatol.*, 37 (2008) 205-212.

- [27] N. Singh, P. Cohen, The T cell in Sjogren's syndrome: Force majeure, not spectateur., in: *J Autoimmun*, 2012, pp. doi:10.1016/j.jaut.2012.1005.1019.
- [28] M. Zeher, R. Adány, G. Nagy, R. Gómez, G. Szegedi, Macrophage containing factor XIII subunit a in salivary glands of patients with Sjögren's syndrome., *J Investig Allergol Clin Immunol.*, 1 (1991) 261-265.
- [29] M. Zeher, P. Surányi, G. Szegedi, CD5 positivity on peripheral blood B lymphocytes in patients with primary Sjögren's syndrome., *Allergol Immunopathol (Madr)*. 18 (1990) 75-78.
- [30] N. Simpson, P. Gatenby, A. Wilson, S. Malik, D. Fulcher, S. Tangye, H. Manku, T. Vyse, G. Roncador, G. Huttley, C. Goodnow, C. Vinuesa, M. Cook, Expansion of circulating T cells resembling follicular helper T cells is a fixed phenotype that identifies a subset of severe systemic lupus erythematosus., *Arthritis Rheum.*, 62 (2010) 234-244.
- [31] C. Zhu, J. Ma, Y. Liu, J. Tong, J. Tian, J. Chen, X. Tang, H. Xu, L. Lu, S. Wang, Increased frequency of follicular helper T cells in patients with autoimmune thyroid disease., *J Clin Endocrinol Metab.*, 97 (2012) 943-950.
- [32] J. Ma, C. Zhu, B. Ma, J. Tian, S. Baidoo, C. Mao, W. Wu, J. Chen, J. Tong, M. Yang, Z. Jiao, H. Xu, L. Lu, S. Wang, Increased frequency of circulating follicular helper T cells in patients with rheumatoid arthritis., in: *Clin Dev Immunol.*, 2012, pp. 827480.
- [33] X. Li, Z. Wu, J. Ding, Z. Zheng, X. Li, L. Chen, P. Zhu, Role of the frequency of blood CD4(+) CXCR5(+) CCR6(+) T cells in autoimmunity in patients with Sjögren's syndrome., *Biochem Biophys Res Commun.*, 422 (2012) 238-244.
- [34] J. Bohnhorst, J. Thoen, J. Natvig, K. Thompson, Significantly depressed percentage of CD27+ (memory) B cells among peripheral blood B cells in patients with primary Sjögren's syndrome., *Scand J Immunol.*, 54 (2001) 421-427.
- [35] A. Hansen, C. Daridon, T. Dörner, What do we know about memory B cells in primary Sjögren's syndrome?, *Autoimmun Rev*, 9 (2010) 600-603.

- [36] A. Hansen, M. Odendahl, K. Reiter, A. Jacobi, E. Feist, J. Scholze, G. Burmester, P. Lipsky, T. Dörner, Diminished peripheral blood memory B cells and accumulation of memory B cells in the salivary glands of patients with Sjögren's syndrome., *Arthritis Rheum.*, 46 (2002) 2160-2171.
- [37] A. Hansen, A. Jacobi, A. Pruss, O. Kaufmann, J. Scholze, P. Lipsky, T. Dörner, Comparison of immunoglobulin heavy chain rearrangements between peripheral and glandular B cells in a patient with primary Sjögren's syndrome., *Scand J Immunol.*, 57 (2003) 470-479.
- [38] W. Dong, P. Zhu, Y. Wang, Z. Wang, Follicular helper T cells in systemic lupus erythematosus: a potential therapeutic target. , *Autoimmun Rev.*, 10 (2011) 299-304.
- [39] C. Ma, S. Suryani, D. Avery, A. Chan, R. Nanan, B. Santner-Nanan, E. Deenick, S. Tangye, Early commitment of naïve human CD4(+) T cells to the T follicular helper (T(FH)) cell lineage is induced by IL-12., *Immunol Cell Biol.*, 87 (2009) 590-600.
- [40] N. Schmitt, R. Morita, L. Bourdery, S. Bentebibel, S. Zurawski, J. Banchereau, H. Ueno, Human dendritic cells induce the differentiation of interleukin-21-producing T follicular helper-like cells through interleukin-12., *Immunity*, 31 (2009) 158-169.
- [41] C. Ma, D. Avery, A. Chan, M. Batten, J. Bustamante, S. Boisson-Dupuis, P. Arkwright, A. Kreins, D. Averbuch, D. Engelhard, K. Magdorf, S. Kilic, Y. Minegishi, S. Nonoyama, M. French, S. Choo, J. Smart, J. Peake, M. Wong, P. Gray, M. Cook, D. Fulcher, J. Casanova, E. Deenick, S. Tangye, Functional STAT3 deficiency compromises the generation of human T follicular helper cells., *Blood*, 119 (2012) 3997-4008.
- [42] A. Vogelzang, H. McGuire, D. Yu, J. Sprent, C. Mackay, C. King, A fundamental role for interleukin-21 in the generation of T follicular helper cells., *Immunity*, 29 (2008) 127-137.

[43] V. Ostiguy, E. Allard, M. Marquis, J. Leignadier, N. Labrecque, IL-21 promotes T lymphocyte survival by activating the phosphatidylinositol-3 kinase signaling cascade., *J Leukoc Biol.*, 82 (2007) 645-656.

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## Tables

	Healthy individuals	Patients with pSS	Patients with EGMs	Patients without EGMs
<b>Number</b>	16	50	25	25
<b>Age (years), mean <math>\pm</math> SD</b>	51.81 $\pm$ 6.22	61.36 $\pm$ 9.65	58.08 $\pm$ 10.17	64.64 $\pm$ 8.03
<b>Sex (male/female)</b>	1/15	2/48	1/24	1/24

**Table 1.** The demographic characteristics of subjects enrolled in the study.

## Figure legends

**Figure 1.** Quantification of circulating T<sub>FH</sub> cells in peripheral blood of pSS patients and healthy individuals. Peripheral blood was acquired from 25 patients with glandular symptoms (B), 25 patients with EGMs (C) and 16 healthy controls (A) then were stained with labelled antibodies as described previously. We used FMO control to determine positive population and all the values were quantified as their percentage in CD4<sup>+</sup> lymphocyte population. On Fig. A-C: Representative dot plots are shown FMO controls which helped to optimize the identification of CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>+</sup>PD-1<sup>+</sup> T cells. Values in the upper right quadrant accord with the percentages of T<sub>FH</sub> cells. Percentages of CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>+</sup> T cells in patients with pSS and controls (D). Percentages of CD4<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>+</sup> T cells in patients with pSS and controls (E). Correlation between CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>+</sup> and CD4<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>+</sup> T cells percentages (F). Percentages of T<sub>FH</sub> cells in patients with pSS and healthy controls (G). Each data point represents an individual subject, on Fig. E. horizontal lines show the median, while on Fig. D. and G. horizontal lines show the mean values. Statistically significant differences are indicated by \*, p < 0.05; \*\*, p < 0.01; \*\*\*, p < 0.001; \*\*\*\*, p < 0.0001; ns, no significant differences.

**Figure 2.** Correlation analysis between lymphocyte populations, antibodies and T<sub>FH</sub> cells in patients with pSS. Correlation between the percentages of peripheral T<sub>FH</sub> cells and CD3<sup>+</sup>CD69<sup>+</sup> early-activated T cells (A) and CD3<sup>+</sup>HLA-DR<sup>+</sup> late-activated T cells (B) and CD4<sup>+</sup> IL-10<sup>+</sup> Tr1 cells (C). Correlation between the percentages of T<sub>FH</sub> cells and serum levels of IgG (D), moreover, between the percentages of T<sub>FH</sub> cells and IgM memory B cells (E) and IgG memory B cells (F) in peripheral blood. Early-activated T cells and late-activated T cells were quantified as their percentage in CD3<sup>+</sup> lymphocyte population, while T<sub>FH</sub> and Tr1 cells

were quantified as their percentage in CD4<sup>+</sup> lymphocyte population. B cell values were calculated as their percentages in CD19<sup>+</sup> lymphocyte population. Correlation between the percentages of T<sub>FH</sub> cells and serum levels of anti-Ro/SSA antibody (**G**) and serum levels of anti-La/SSB antibody (**H**). The frequency of peripheral T<sub>FH</sub> cells in pSS patients and controls compliance with the anti-Ro/SSA and anti-La/SSB antibody (**I**). Each data point represents an individual subject, horizontal lines show the median values. \*, p < 0.05; \*\*, p < 0.01; \*\*\*, p < 0.001; ns, no significant differences.

**Figure 3.** Increased levels of serum soluble cytokines in patients with pSS. The percentages of peripheral T<sub>FH</sub> cells according to the levels of plasma IL-12 (**A**) and IL-21 (**B**) cytokine in pSS patients and healthy controls. The percentages of peripheral T<sub>FH</sub> cells within the IL-21 negative and positive-group according to the patients symptoms (**C**). Each data point represents an individual subject, horizontal lines show the median values. \*, p < 0.05; \*\*, p < 0.01; ns, no significant differences.

**Figure 4.** Correlation analysis between the percentages of peripheral T<sub>FH</sub> cells and the focus scores of labial salivary gland biopsies in patients with pSS. T<sub>FH</sub> cells were quantified as their percentage in CD4<sup>+</sup> lymphocyte population. Each data point represents an individual subject.

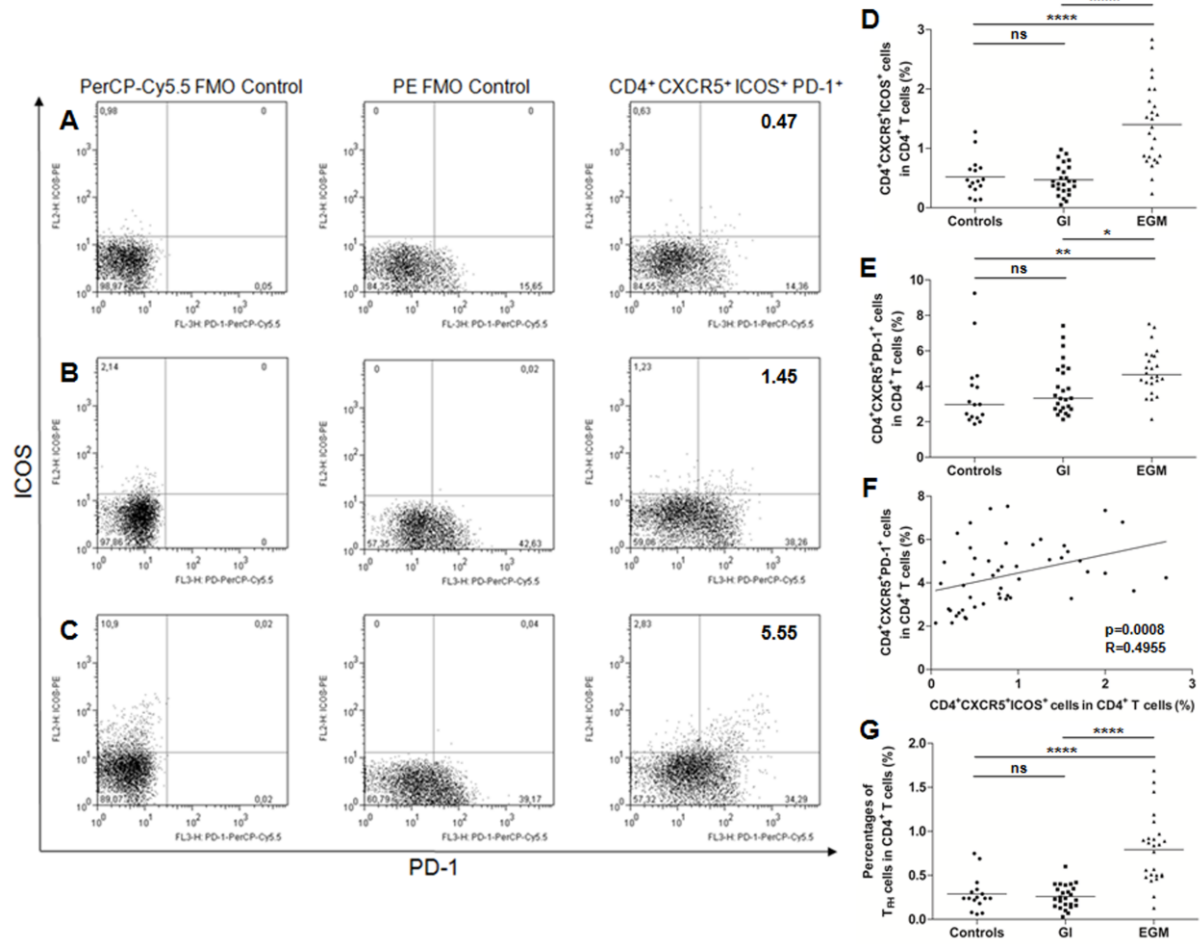


Figure 1



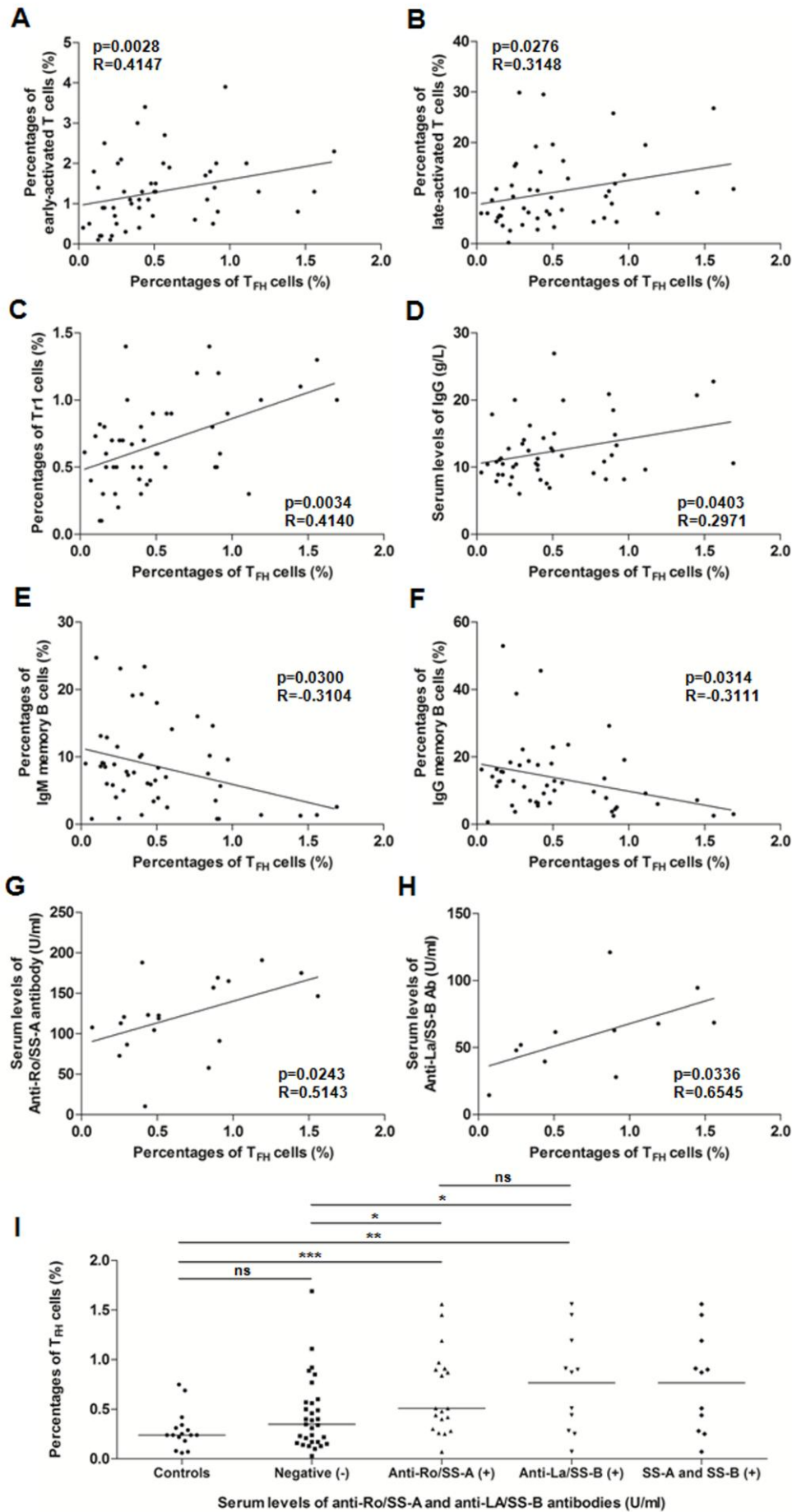


Figure 2

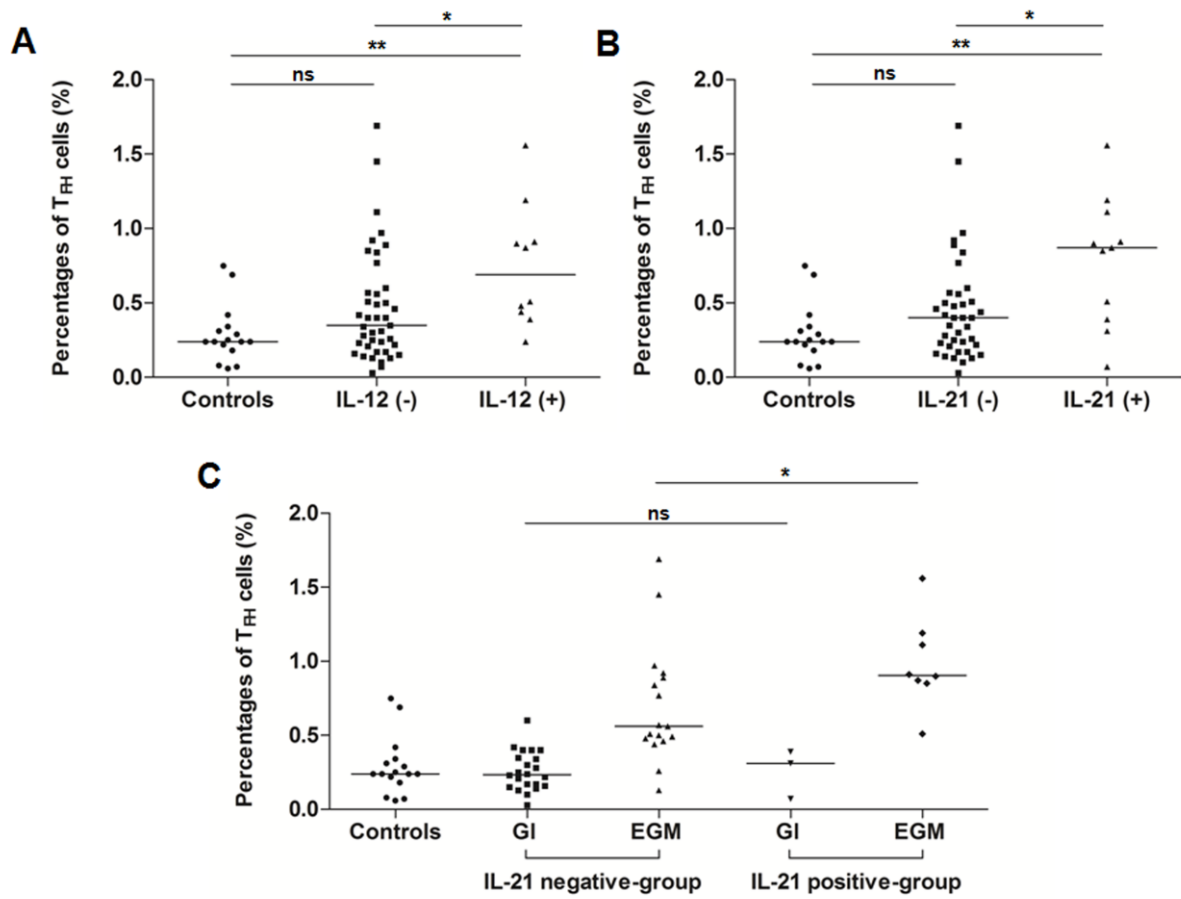


Figure 3

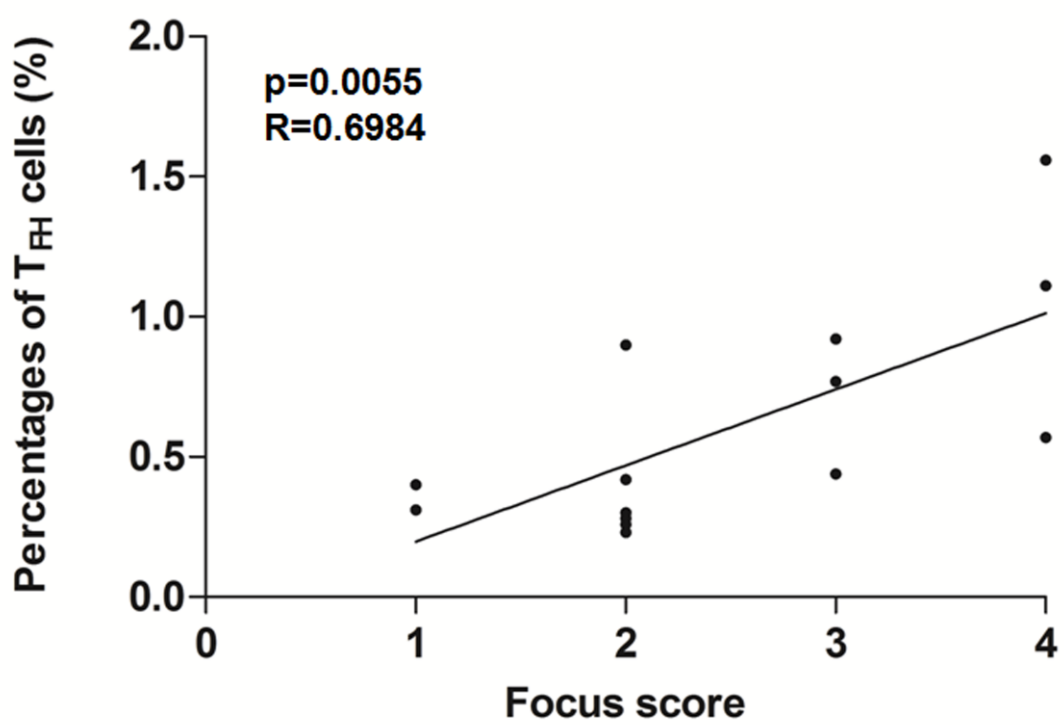


Figure 4

**Highlights**

- Increased percentages of T<sub>FH</sub> cells are associated with the severity of pSS.
- T<sub>FH</sub> cell percentages correlates with the enhanced counter-regulatory activity.
- T<sub>FH</sub> cell percentages correlates with certain B cell subsets and autoantibody levels.
- IL-12 and IL-21 have an important role in immune processes regulated by T<sub>FH</sub> cells.

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