

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

Investigation of the pathological role of follicular T helper cells
in autoimmune diseases

by Krisztina Szabó

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DEBRECEN, 2016

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The Examination takes place at the Life Science Building room number 3.506, Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Debrecen at 11:00 a.m. on 22th of March, 2016

Head of the **Defense Committee:** László Fésüs, MD, PhD, DSc, MHAS

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen at 1:00 p.m. on 22th of March, 2016

I. INTRODUCTION

Extensive examination of both innate and adaptive arms of the immune system has provided a number of new findings for the scientific literature. Nowadays, since with the understanding of the pathomechanism of immunological diseases, cells that are responsible for regulating basic immune processes have become the aims of research and they may be excellent therapeutic targets in the future. For several decades, comprehensive research and high-level treatment of systemic autoimmune diseases, with particular attention to the investigation of primary Sjögren's syndrome (pSS) and systemic lupus erythematosus (SLE), are engaged in the Division of Clinical Immunology, Faculty of Medicine, University of Debrecen. In our study, we analyzed comprehensively the abnormal function of the components of adaptive immune system in the peripheral as well as in the inflammatory environment. However, collective literature and system-level summary about the relationship between follicular T helper (T_{FH}) cells and B cell subsets in either disease is not yet available. This encouraged us to investigate the role of these cells in systemic autoimmune diseases.

II. BACKGROUND

II.1. The general features of autoimmune diseases

Based on past years of immunopathological research, we know now that the background of many chronic inflammatory and tissue damage associated diseases is abnormal autoimmunity. Autoimmune processes also occur under physiological conditions, however due to the well-regulated immune responses they do not endanger the safety of the organism. Autoimmune diseases are multifactorial diseases which develop on the ground of genetic "predisposing" factors, epigenetic modifications, environmental and hormonal influences. In genetically susceptible individuals, tolerance that evolved against their own structures or mechanism which induces and promotes immune tolerance are impaired and leads to the formation of the disease.

II.1.1. Sjögren's syndrome

The pSS is a chronic inflammatory disease affecting approximately 0.6% of the population, thus considered one of the most common systemic autoimmune disorders. It can occur at any age, but it is developed particularly in menopausal period, in women around age between 40-60 years. The disease represents the highest female dominance among other autoimmune

disorders with a female to male ratio of 9:1. The condition can be mainly defined by the damage and consequential loss of function of the exocrine glands leading to the development of characteristic symptoms of the disease. Glandular symptoms are the first that occur in pSS, the involvement of the lacrimal glands causes dry eyes, while salivary dysfunction leads to dry mouth. In addition to gland-related symptoms, extraglandular manifestations (EGMs) can be developed during the course of the disease as well. The pathogenesis of pSS is still not fully understood, but it is certain that its development is due to a multi-step process. Changes initiated by intrinsic and extrinsic factors leads to the intensification of autoimmune processes and the damage of exocrine glands. These histological alterations can be examined predominantly in labial salivary gland biopsy samples. The damage and dysfunction of the epithelium in the salivary gland tissue results the expression and production of chemokines (CXCL9, CXCL10), adhesion molecules (ICAM-1), activation markers (MHCI, CD40) and cytokines (IL-6, TNF- α), which promotes the infiltration of mononuclear cells. The influx of DCs (pDC, mDC) into the inflammatory microenvironment initiates the activation of NK cells and T cells. These circumstances could enhance on the current abnormal immune processes. With the secretion of BAFF, the adaptive immune system is activated and it leads to the development of the impaired humoral immune response which is the characteristic feature of the disease. The formation of ectopic germinal centers (GCs) is the result of enhanced local B cell activity in the labial salivary gland biopsies which can provide an excellent opportunity for the investigation of autoreactive mechanism; moreover it has a predictive value for forecast subsequent malignant lymphomas in the future.

II.1.2. Systemic lupus erythematosus

SLE is a chronic systemic autoimmune disease with heterogeneous symptoms. Inflammatory processes can be affected numerous organs, but mostly it is typical in the skin, joints, kidneys and the central nervous system. Firstly, it is developed in conceiving women but it can be occur in any age group. Like in pSS, a strong female dominance is also typical for the disease. The disease course is variable; active and inactive stages are followed each other. Due to the intensive research, a number of information assembled regarding the pathogenesis of the disease, but the details are still not fully understood. The initial step of the disorder is the necrotic and apoptotic processes which triggered by external environmental factors. In the latter case, the disturbed cell death leads to the damage of the membrane integrity on the blebs and their content escape into the extracellular space. As the result of these processes, the double stranded (ds) DNA, RNA and chromatin particles which are characteristics for SLE,

are recognized by the immune system's own cells and induce an autoimmune process. Another source of autoantigens can be the "NETosis" used by neutrophils. During this mechanism, these cells emit DNA and ribonucleoprotein containing structures into the intercellular space. Whether the above mentioned process is failed, the emitted autoantigens can trigger the immune cells. Cell debris activates mDCs, while immune complexes and NETs can be recognized by pDCs. The adaptive immune system is linked to the pathogenesis of SLE through BAFF secretion by mDCs and pDCs or mDC activated CD4⁺ T cells. Antibody producing plasma cells are developed by supporting the survival and differentiation of autoreactive B cells. The most important manifestation of SLE is the deposition of immune complexes (ICs) in the kidney. The existence of abnormal B cell responses is supported by those studies in which BAFF molecule and ectopic GC have been shown in renal biopsies in patients with lupus nephritis.

II.1.3. The origin and function of conventional and circulating follicular T helper cells

In recent years, the most intensively studied cell type is the T_{FH} cell, which has a central role in the GC reactions. Recently published data shed light on that T_{FH} cells are different from T helper (Th)1/Th2/Th17 cell lines in their gene expression and transcriptional program as well as function, and express a number of factor which are also effective in supporting B-cell immune responses. The differentiation of T_{FH} cells is a multistep process; the first phase occur between DCs and naive CD4⁺ T cells in the T cell zone of secondary lymphoid tissues. The basic condition of the development toward T_{FH} cell lines is the increased expression of characteristic Bcl-6 cell transcription factor and the decreased level of Blimp-1. Antigens presented by DCs enhance the expression of BATF through costimulatory signals (B7-CD28, CD40, CD40L) and cytokines (IL-6, IL-12, IL-27) in CD4⁺ T cells which then activates the Bcl-6 and c-maf transcription factors. On the surface of CD4⁺ T cells, the expression of CXCR5 (B cell zones chemokines), CD40L, inducible costimulatory (ICOS) receptor, programmed cell death 1 (PD-1), signal transmitter lymphocyte activator molecule [SLAM]-associated protein (SAP) are increased and the secretion of CXCL13 and IL-21 are also enhanced. During this process, cells migrate to the T-B cell border. The next stage of the development is the interaction of pre-T_{FH} cells and activated B cells. The two cell types are met at the border areas, they contact with each other effecting their further activation via signals, which results the development of extrafollicular short-lived plasma cell mediated antibody responses or the support of the survival and differentiation of B cells in secondary follicles. In the GC's dark zone, B-cell receptor (BCR) on the surface of dividing centroblasts

go through somatic hypermutation (SHM), then the cells transformed into centrocytes and migrate to the light zone. Here, B cells undergo a maturation and selection process with the collaboration of terminally differentiated T_{FH} cells and FDCs. As a result, high-affinity antibody producing, isotype switched long-lived plasma cells and memory B cells are generated. In recent years, the circulating, so called T_{FH} -like cells have become in focus. In terms of their origin, it is assumed that they are memory cells and their differentiation begins in the T_{FH} cell line, but they leave the GC before they reach the fully mature state of the development. The circulating $CD4^+CXCR5^+$ memory T_{FH} cells also express ICOS and PD-1 molecules, however, in a much lower level than conventional T_{FH} cells. They are also able to promote B cell responses, but not as effective as GC T_{FH} cells. These cells are forming a heterogeneous group at the periphery, which can be further classified based on the type of memory T cells and the expression of co-stimulatory and chemokine receptors as well. According to the literature, in terms of their function and the genetic profile, circulating T_{FH} -like cells that correspond to $ICOS^+PD-1^+CCR7^{int} T_{FH2}$ or T_{FH17} are the most similar to GC T_{FH} cells.

II.2. Aim of the study

Laboratory parameters in pSS and SLE, and the fact that ectopic GCs can be detected in the target organ with immunohistological examination of both diseases, are supported the abnormal mechanisms of adaptive immunity. The role of T_{FH} cells as the regulatory element of the humoral immune responses or their relationship with other immune cells, laboratory data and clinical symptoms have not been studied yet. The importance of the research is highlighted by the fact that we had the opportunity to investigate the glandular or systemic forms of pSS as well as the active and inactive stadium of SLE. In our work we have formulated the following objectives:

- the determination of the percentage of T_{FH} -like cells in pSS patient with glandular or systemic symptoms as well as in patients with SLE with inactive and active disease, moreover we compare the received results with the parameters of healthy individuals
- diagnostic profile the immune cells in patients with pSS and the comparison of the received results to T_{FH} cell-like cells
- the investigation of patient's laboratory parameters, namely serum Ig subgroups, characteristic autoantibodies, RF, ICs and complement components and examining the correlation between the obtained results and cell studies

- the examination of B cell subpopulations according to the classification of the groups of patients based on clinical symptoms and their comparison to control data
- obtain labial salivary gland biopsy sample from previously measured patients with pSS and examine them with conventional and immunohistochemistry methods, investigate whether T_{FH} cells are involved in the formation of tertiary lymphoid neogenesis and examine the correlation between histological findings, laboratory parameters and clinical symptoms.

III. PATIENTS AND METHODS

III.1. Patients

III.1.1. Ethical statement

Blood samples from patients with pSS and SLE were used for serological and cellular studies at the Division of Clinical Immunology, Faculty of Medicine, University of Debrecen. Histological investigations were performed on labial salivary gland biopsy samples obtained from the archives of the Department of Pathology, Faculty of Medicine, University of Debrecen. Informed written consent was obtained from all subjects, and the study was approved by the Ethics Committee of the University of Debrecen (Reference Number: IX-R-052/00016-22/2012). All experiments carried out were in compliance with the Declaration of Helsinki.

III.1.2. Patients with pSS enrolled in the peripheral study

Total of 75 patients with pSS (72 female and 3 male; mean age: 60.20 ± 9.58 years) were enrolled in the study. The diagnosis of pSS was based on the European-American consensus criteria. Among pSS patients, 40 suffered from EGMs, while 35 had only glandular symptoms. The distribution of EGMs of pSS patients were as follows: polyarthrititis n=25, polyarthralgia n=11, Raynaud's phenomenon n=18, lymphadenopathia n=3, vasculitis n=5, myositis n=1, polyneuropathia n=2. The control group consisted of 37 age- and sex-matched (35 female and 2 male; mean age: 45.58 ± 12.99 years) healthy volunteers. No patients or controls enrolled in this study had ongoing infections, either viral or bacterial.

III.1.3. Patients with pSS enrolled in the histological study

In the present study, we enrolled ten female patients (mean age \pm SD: 57.2 ± 11.4) with pSS. The diagnosis of the patients was confirmed with positive LSG biopsy at the disease onset.

Data were obtained retrospectively from their records which contained detailed information on symptoms, physical conditions, laboratory and other findings.

III.1.4. Patients with SLE enrolled in the peripheral study

Total of 25 patients with SLE (24 female and 1 male; mean age: 41.17 ± 13.20 years) were enrolled in the study. All patients with SLE fulfilled the corresponding diagnostic criteria for lupus, and their disease activity was assessed by the SLE Disease Activity Index (SLEDAI). SLE patients were classified according to having inactive or active disease status: SLEDAI<6 group comprised subjects with inactive disease (n=17), while SLEDAI \geq 6 group consisted of subjects with active disease (n=8). All of the SLE patients received per os methylprednisolone therapy with an average dose of 4 mg daily. The control group consisted of 21 age- and sex-matched (20 female and 1 male; mean age: 39.10 ± 12.43 years) healthy volunteers.

III.2. Laboratory methods

III.2.1. Determination of lymphocyte subpopulations

For phenotypic analysis from heparinized blood samples we used monoclonal antibodies against the following cell surface markers: CD3, CD4, CD8, CD19, CD16 and CD56 (BD Biosciences). The expression of T-lymphocyte activation markers such as anti-CD69 and anti-HLA-DR were also determined on CD3⁺ cells (BD Biosciences). For identification naive and memory B cells we used IgD/CD27/CD19 (Beckman Coulter). Samples were processed according to the Coulter Q-PREP protocol and system. For the identification of CD4⁺ T helper cell subsets we used intracytoplasmic cytokine staining method. Briefly, 1 ml whole heparinized blood was diluted 1:1 in RPMI-1640 GLUTAMAXTM-I supplemented with 100 U/ml penicillin, 100 ng/ml streptomycin and 10% FCS (Life Technologies). Cells were stimulated using 25 ng/ml phorbol–myristate–acetate (PMA) and 1 ng/ml ionomycin (Sigma Aldrich) for 4 h at 37°C in an atmosphere containing 5% CO₂. The transport of *de novo* synthesized cytokines from the Golgi apparatus was inhibited by 10 mg/ml brefeldin-A (Sigma Aldrich). Monoclonal antibodies against the following cell surface molecules were used: CD4, CD8, IFN- γ , IL-4, IL-17 and IL-10 (all from BD Biosciences). The cells then fixed and permeabilized with IntraprepTM permeabilization reagent (Beckman Coulter) according to the manufacturer's instructions. Measurements were performed on a Coulter FC500 flow cytometer (Beckman Coulter).

III.2.2. Analysis of B cell subsets by flow cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinised venous blood sample by Ficoll-Histopaque (Sigma-Aldrich) density-gradient centrifugation. For identification of naive and memory B cell subsets, we used the combination of IgD/CD27/CD19 (all from Beckman Coulter Inc). To identify naive, mature-naive, primarily memory and transitional B cell subpopulations; cells were stained with the following combination of monoclonal antibodies: CD38/CD27/CD19/CD24 (BD Biosciences, Beckmann Coulter and BioLegend). The stained cells were measured by FACS Calibur flow cytometer (Becton Dickinson) and data were analysed using FlowJo Software (Treestar).

III.2.3. Assessment of circulating T_{FH} cell ratio by flow cytometry

For the identification of circulating T_{FH} cells we used whole blood (50 patients with pSS) or PBMCs (25 patients with pSS and 25 patients with SLE). For the assessment of circulating T_{FH} cells we used the combination of CXCR5/ICOS/PD-1/CD4 (all from BD Biosciences) monoclonal antibodies. For the determination of naive and activated or memory $CD4^+$ T cell subsets we used CD45RA reagent (BioLegend). Measurements were performed and data were collected by FACS Calibur flow cytometer and data were analysed using FlowJo Software.

III.2.4. Analysis of IL-21R expression of B cells in pSS

For the measurement of $CD19^+IL-21R^+$ cells, we used PBMCs. Cells were stimulated using 6,5 μ g/ml anti-human B cell receptor (BCR) antibody (Jackson ImmunoResearch, West Grove, PA, USA) and 50 ng/ml recombinant human IL-21 (R&D Systems, Inc., Minneapolis, MN, USA) for 18 hours at 37°C in an atmosphere containing 5% CO_2 . For the evaluation of IL-21R expression, anti-CD19 and anti-IL-21R antibodies were used (Beckman Coulter and BioLegend), and mean fluorescence intensity (MFI) was determined. The measurements were performed on a FACS Calibur flow cytometer (Becton Dickinson).

III.2.5. Intracellular cytokine measurement by flow cytometer

The cytoplasmic IL-21 content of circulating T_{FH} cells was also determined by flow cytometry. Briefly, isolated PBMCs were cultured at a concentration of 2×10^6 /ml in 24-well tissue culture plates for analysing single-cell cytokine production. Total PBMCs were incubated with PMA (25 ng/ml), ionomycin (1 μ g/ml), and Golgi Stop brefeldin-A (10 μ g/ml) (all from Sigma Aldrich) for 5 h at 37°C in 5% CO_2 milieu. Cell surface staining was performed with a cocktail of CXCR5/PD-1/CD4 monoclonal antibodies and intracellular

cytokines were stained with anti-IL-21. For intracellular IL-10 induction of B cells, PBMCs were stimulated with CpG (ODN 2006 type B; 0.5 μ M/ml; Hycult Biotech) for 48 h at 37°C in 5% CO₂ milieu. For the last 5 h, PMA (25 ng/ml), ionomycin (1 μ g/ml), and Golgi Stop brefeldin-A (10 μ g/ml) were added to the culture. Cells were then stained with anti-CD19-PE-Cy5. Intracellular staining method was performed with IL-10-PE. In both cases, the cells then fixed and permeabilized with IntraprepTM permeabilization reagent (Beckman Coulter) according to the manufacturer's instructions. Measurements were performed and data were collected by FACS Calibur flow cytometer and data were analysed using FlowJo Software.

III.2.6. Determination of serum soluble cytokines

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

III.2.7. Evaluation of serological parameters

The level of serum immunoglobulins (IgG, IgA and IgM), complement components (C3, C4) is measured by BN II Nefelometer (Siemens AG) with the help of calibration reagents (DIALAB). The determination of circulating ICs was performed by polyethylene glycol precipitation. As part of the routine diagnostic evaluation, autoantibodies were determined by ELISA technique with AUTOSTAT II kits (Hycor Biomedical) according to the manufacturer's instructions.

III.2.8. LSG samples and conventional histological analysis

Formalin-fixed, paraffin-embedded (FFPE) tissue blocks were obtained and 4 μ m thick serial sections of LSG tissue specimens were prepared and stained with haematoxylin-eosin (HE) for conventional histopathological examination. The focus score (FS) was defined as the group of inflammatory cell aggregates containing at least 50 mononuclear cells per 4 mm² of tissue area. It was classified as FS=0: no lymphocytic infiltration; FS=1: less than 1 lymphocytic focus per 4 mm²; FS=2: less than 2 lymphocytic foci per 4 mm²; FS=3: two or more lymphocytic foci per 4 mm².

III.2.9. Immunohistochemical analysis

Immunohistochemical (IHC) staining was performed on serial sections of FFPE tissue blocks using standard methods. We used monoclonal antibodies against the following molecules: CD4, CD5, CD20, CD138, PD-1, CD84 and Bcl-6. The slides were incubated with primary antibodies for 1 hour at room temperature. Biotin-free EnvisionTM/HRP (Dako) system as secondary Ab with Very Intense Purple (VIP) peroxidase substrate (Vector Laboratories) were used for detection. The sections were then counterstained with methyl green (Vector Laboratories). Image analysis was performed by Panoramic Viewer software 1.15.2. (3D-Histech).

III.2.10. The characterization of periductal infiltrates

The organizational levels of each lymphocytic infiltrate were graded by IHC staining of serial sections using CD4 and CD20 cell markers. A small number of distributed perivascular and intraepithelial lymphocytes were graded as 1. Mild lymphocytic aggregates without clear organization of separate T and B cell zones were defined as grade 2. More organized lymphoid follicles were classified as grade 3. Aggregates with the highest level of arrangement, which displayed distinct T and B cell regions, were graded as 4.

III.2.11. Double immunofluorescence staining

Double immunofluorescence (IF) staining for Bcl-6 in combination with CD3 or CD20 was carried out with sequential immunostaining on FFPE sections. After 1 hour treatment with anti-Bcl-6 primary Ab, the slides were incubated using biotin-free EnvisionTM/HRP (Dako) and followed by a TMR-conjugated tyramid reagent of the fluorescent amplification kit (Perkin Elmer Life Science) to visualize the red nuclear fluorescence. The second layer of the double IF staining was applied with anti-CD3 or anti-CD20 primary Abs plus biotinylated anti-mouse secondary IgG F(ab')₂ followed by streptavidin-FITC. Nuclear counterstaining was made with DAPI (Vector Laboratories). Images were obtained using a Zeiss AXIO Imager Z2 microscope (Carl Zeiss Microscopy) equipped with the following objectives: 10x/0.3 NA; 20x/0.5 NA. For transferring and editing images, Isis software (MetaSystems Group Inc., Newton, MA, USA) and Adobe Photoshop CS5 version 12.0 were used.

III.3. Statistical analysis

Data were represented and statistical analysed with GraphPad Prism 5 software (Graphpad Software). To assess the distribution of the data Shapiro-Wilk normality test were used. In

cases of normal distribution, if the F probe was granted we used unpaired t test, otherwise it was not granted we used unpaired t test with Welch's correction 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. The correlations between two variables were evaluated with Pearson's correlation coefficient, while in cases of non-normal distribution, Spearman's test was used. Differences were considered statistically significant at $p < 0.05$.

IV. RESULTS

IV.1. Results in peripheral measurements

IV.1.1. Quantification of circulating T_{FH}-like cells in pSS from whole blood samples

In the first part of our study, we determined the percentages of CD4⁺CXCR5⁺ICOS⁺PD-1⁺ T_{FH}-like cells in the peripheral blood of 50 patients with pSS. We found significant higher percentages of T_{FH}-like cells in patients with EGM than in patients with glandular symptoms only and healthy controls ($0.79\% \pm 0.39$ vs $0.29\% \pm 0.19$, respectively, $p < 0.0001$ and $0.79\% \pm 0.39$ vs $0.26\% \pm 0.13$, respectively, $p < 0.0001$). The proportions of T_{FH}-like cells in patients without EGMs did not differ from control values. 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_{FH}-like cells. When we analyzed the percentages of T_{FH}-like cells in patients with pSS with emphasis on the presence of autoantibodies, the percentages of T_{FH}-like cells showed one and a half-fold increase compared to antibody-negative group (0.510 (0.070 - 1.560) % vs. 0.350 (0.030 - 1.690) %, respectively, $p = 0.0341$). Regarding anti-La/SSB, we found significant 2.5-fold higher percentages, compared to antibody-negative group (0.870 (0.070 - 1.560) % vs 0.350 (0.030 - 1.690) %, respectively, $p = 0.0452$).

IV.1.2. Increased levels of serum soluble cytokines in patients with pSS

We also examined the relationship between the concentrations of serum IL-12, IL-21 and the percentages of T_{FH}-like cells. The percentages of T_{FH}-like cells showed a significant 3-fold increase in IL-12-positive group compared to healthy controls (0.69 (0.24 - 1.56) % vs. 0.24 (0.06 - 0.75) %, respectively, $p = 0.0011$) and a significant 2-fold increase between the positive and negative groups (0.69 (0.24 - 1.56) % vs. 0.35 (0.03 - 1.69) %, respectively, $p = 0.0249$). The percentages of T_{FH}-like cells showed a significant 3,5-fold increase in IL-21-positive group

compared to healthy controls (0.87 (0.07-1.56) % vs. 0.24 (0.06-0.75) %, respectively, $p=0.0017$) and a significant 2-fold increase between the IL-21-positive and IL-21-negative groups (0.87 (0.07-1.56) % vs. 0.40 (0.03-1.69) %, respectively, $p=0.0186$). 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_{FH} -like cells and the presence of EGMs. The percentages of T_{FH} -like 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 (0.560 (0.130-1.690) % vs. 0.905 (0.510-1.560) %, respectively, $p=0.0414$).

IV.1.3. Quantification of circulating T_{FH} -like cells in pSS and SLE from PBMCs

Further investigations with T_{FH} -like cells were performed on PBMCs in patients with pSS and SLE. We handled the pSS group separately from the previous population. According to our results, the percentages of $CD4^+CXCR5^+ICOS^+PD-1^+T_{FH}$ -like cells were significantly increased in pSS patients with EGMs when compared to pSS patients with glandular symptoms and healthy controls ($0.4121 \pm 0.2753\%$ vs. $0.2253 \pm 0.1238\%$, respectively, $p=0.0322$ and $0.4121 \pm 0.2753\%$ vs. $0.2235 \pm 0.0979\%$, respectively, $p=0.0218$). The frequency of T_{FH} -like cells was also elevated in SLE, however this difference was significant only when the overall SLE patients were compared with the controls ($0.3369 \pm 0.2029\%$ vs. $0.2235 \pm 0.0979\%$, respectively, $p=0.0184$). Regarding anti-dsDNA antibody-positive group, we found a significant 1.6-fold increase compared to control values ($0.3717 \pm 0.2153\%$ vs. $0.2235 \pm 0.0979\%$, respectively, $p=0.0192$).

IV.1.4. Determination of IL-21 producing T_{FH} -like cells in pSS and SLE from PBMCs

To establish the cytokine profile of circulating T_{FH} -like cells, we determined intracellular IL-21 cytokine production. We found that the percentages of $CD4^+CXCR5^+PD-1^+IL-21^+$ T cells were significantly higher in pSS patients with EGMs than control values ($0.2449 \pm 0.1657\%$ vs. $0.1469 \pm 0.0649\%$, respectively, $p=0.0442$). When we measured the percentages of T_{FH} cells in patients with pSS with an emphasis on the presence of autoantibodies, the percentages of T_{FH} cells showed a significant 1.8-fold increase in anti-Ro/SSA antibody-positive pSS group compared to controls ($0.2742 \pm 0.1418\%$ vs. $0.1469 \pm 0.0649\%$, respectively, $p=0.0297$). The ratio of IL-21 producing T_{FH} -like cells was significantly elevated in the overall SLE group, and in both SLE subgroups (SLEDAI<6 and SLEDAI>6 groups) compared to those measured in controls ($0.3125 \pm 0.1886\%$ vs. $0.1469 \pm 0.0649\%$, respectively, $p=0.0003$,

0.2786 \pm 0.1805% vs. 0.1469 \pm 0.0649%, respectively, p=0.0100 and 0.3846 \pm 0.1968% vs. 0.1469 \pm 0.0649%, respectively, p=0.0123). In SLE patients, we found significant 2.5-fold higher percentages of T_{FH} cells in anti-dsDNA antibody-positive SLE group compared to healthy controls (0.3638 \pm 0.1982% vs. 0.1469 \pm 0.0649%, respectively, p=0.0009); additionally, 1.5-fold higher ratios of T_{FH} cells compared to anti-dsDNA antibody-negative SLE group (0.3638 \pm 0.1982% vs. 0.2356 \pm 0.1509%, respectively, p=0.0489).

IV.1.5. Phenotypic measurement of circulating T_{FH}-like cells

We also determined CD45RA⁺ naive CD4⁺ T cells and CD45RA⁺ activated or memory CD4⁺ T cells in a smaller group of patients and controls. Naive CD45RA⁺ T cells mostly showed reduced ICOS and PD-1 expression compared to activated or memory CD45RA⁺ T cells.

IV.1.6. Distribution of peripheral B cell subpopulations in pSS and SLE from PBMCs

According to the expression of IgD, CD27, CD38 and CD24 cell surface markers, the following B cell subsets were identified: CD19⁺IgD⁺CD27⁻ naive B cells, CD19⁺IgD⁺CD27⁺ non-switched memory B cells, CD19⁺IgD⁻CD27⁺ switched memory B cells, CD19⁺IgD⁻CD27⁻ double negative (DN) B cells, CD19⁺CD38⁻CD24^{hi}CD27⁺ primarily memory B cells, CD19⁺CD38^{hi}CD24^{hi}CD27⁻ transitional B cells, CD19⁺CD38⁺CD24⁺ mature-naive B cells and CD19⁺CD38^{hi}CD27^{hi} plasmablasts. Cells were quantified as their percentage in the CD19⁺ lymphocyte population. Regarding CD19⁺IgD⁻CD27⁺ B cells, percentages were decreased in the overall pSS patient population compared to values in healthy individuals (15.07 \pm 7.65% vs. 23.23 \pm 6.78%, respectively, p=0.0005). On the contrary, B cell percentages in the total SLE patient population and controls were similar. However, SLE patients with SLEDAI<6, unlike patients with SLEDAI>6, had significantly decreased CD19⁺IgD⁻CD27⁺ B cell percentages compared to controls (17.53 \pm 13.89% vs. 23.23 \pm 6.78%, respectively, p=0.0321). The ratio of CD19⁺IgD⁺CD27⁺ B cells was significantly lower in the whole SLE group than in healthy individuals (10.98 \pm 10.60% vs. 21.64 \pm 11.52%, respectively, p=0.0019). Peripheral CD19⁺IgD⁻CD27⁻ DN B cell proportions were significantly reduced in the overall pSS patients group set against to control values (3.214 \pm 2.463% vs. 3.796 \pm 1.681%, respectively, p=0.0290). In contrast to measurements in pSS, the percentages of DN B cells were significantly heightened in whole SLE group than in healthy individuals (6.906 \pm 4.525% vs. 3.796 \pm 1.681%, respectively, p=0.0119). The percentages of CD19⁺IgD⁺CD27⁻ B cells were significantly increased in the whole pSS patient group compared to controls (63.87 \pm 20.76% vs. 51.32 \pm 15.14%, respectively, p=0.0261).

Frequency of CD19⁺IgD⁺CD27⁻ B cells was also significantly elevated in the total SLE patient group ($62.88 \pm 21.87\%$ vs. $51.32 \pm 15.14\%$, respectively, $p=0.0471$). The percentages of CD19⁺CD38⁻CD24^{hi}CD27⁺ B cell ratio was significantly decreased in pSS patients without EGMs when compared to healthy subjects ($23.29 \pm 9.827\%$ vs. $34.58 \pm 13.91\%$, respectively, $p=0.0289$). The frequency of CD19⁺CD38⁻CD24^{hi}CD27⁺ B cells were significantly reduced in the whole SLE patient group and SLEDAI>6 group, compared to control values ($22.77 \pm 14.45\%$ vs. $34.58 \pm 13.91\%$, respectively, $p=0.0074$ and $17.10 \pm 12.11\%$ vs. $34.58 \pm 13.91\%$, respectively, $p=0.0042$). There was no difference in the pSS groups regarding the distribution of both CD19⁺CD38^{hi}CD24^{hi}CD27⁻ B cells. On the other hand, we found that the ratio of transitional B cells was significantly elevated in pSS patients with the presence of anti-Ro/SSA antibody compared to subject whom autoantibody levels were under the threshold value ($9.842 \pm 7.766\%$ vs. $3.722 \pm 2.320\%$, respectively, $p=0.0499$). In the overall SLE group and in patients with SLEDAI>6, the frequency of CD19⁺CD38^{hi}CD24^{hi}CD27⁻ B cells was significantly higher than control values ($10.35 \pm 7.78\%$ vs. $5.30 \pm 2.42\%$, respectively, $p=0.0045$ and $12.71 \pm 7.73\%$ vs. $5.30 \pm 2.42\%$, respectively, $p=0.0323$). The ratio of CD19⁺CD38⁺CD24⁺ B cells was also significantly elevated not only in the total SLE group, but in the SLEDAI>6 group compared to those measured in healthy subjects ($35.86 \pm 14.86\%$ vs. $27.74 \pm 9.33\%$, respectively, $p=0.0297$ and $39.32 \pm 11.61\%$ vs. $27.74 \pm 9.33\%$, respectively, $p=0.0094$). The ratio of peripheral CD19⁺CD38^{hi}CD27^{hi} plasmablasts showed a different tendency in the two disease. The percentages of these cells were significantly lower in the overall group of pSS patient, as well as in the patients with EGMs groups than control values ($0.134 \pm 0.154\%$ vs. $0.267 \pm 0.293\%$, respectively, $p=0.0050$ and $0.127 \pm 0.183\%$ vs. $0.267 \pm 0.293\%$, respectively, $p=0.0014$). However, in patients with SLE, we found significantly higher frequency of CD19⁺CD38^{hi}CD27^{hi} plasmablasts only in SLEDAI>6 group compared to healthy subjects ($1.150 \pm 1.181\%$ vs. $0.2671 \pm 0.2932\%$, respectively, $p=0.0403$).

IV.1.7. IL-21 receptor expression is enhanced on CD19⁺ B cells in pSS

We observed significantly enhanced IL-21R expression on CD19⁺ B cells in the whole group of pSS patients (mean \pm SD: 3.434 ± 0.929 MFI vs. 2.961 ± 0.585 MFI, respectively, $p=0.0288$), as well as in subgroup of patients with EGMs, compared to values measured in healthy individuals (mean \pm SD: 3.578 ± 0.901 MFI vs. 2.961 ± 0.585 MFI, respectively, $p=0.0195$).

IV.1.8. Assessment of IL-10 producing B cells in pSS and SLE

As a next step, we cultured PBMCs for 5 h or 48 h, stimulated them with PIB alone or in combination with CpG, and then determined the ratios of IL-10 producing CD19⁺ B cells. The total frequency of IL-10 producing CD19⁺ B cells including B10 and matured B10_{PRO} cells, was significantly elevated compared with PIB alone treated cells in case of each equivalent groups (control: 7.335% \pm 0.9034 vs. 3.675% \pm 1.050; p=0.0384; pSS: 5.838% \pm 0.6618 vs. 2.748% \pm 1.032; p=0.0357; SLE: 5.477% \pm 0.6775 vs. 1.907% \pm 0.4094; p=0.0007). However, there were no significant differences between the patients groups and healthy controls regarding the distribution of IL-10⁺CD19⁺ B cells after 48 h of CpG stimulation.

IV.1.9. Correlation analysis between peripheral T_{FH} cells and T lymphocyte populations in patients with pSS

We analyzed the possible associations between the percentages of T_{FH} cells and other investigated lymphocyte populations. We found positive correlations between the proportions of early-activated T cells and T_{FH} cells (R=0.4147, respectively, p=0.0028), the percentages of late-activated T cells and T_{FH} cells (R=0.3148, respectively, p=0.0276) as well as the proportions of Tr1 cells and T_{FH} cells (R=0.4140, respectively, p=0.0034). There was no significant correlation between T_{FH} cells and the other investigated T lymphocyte subpopulations.

IV.1.10. Correlation analysis between peripheral T_{FH} cells and B lymphocyte populations in pSS patients with EGMs

When we focused only on pSS patients with EGMs, we revealed strong significant positive correlations between IL-21 producing T_{FH} cells and CD19⁺CD38^{hi}CD24^{hi}CD27⁻ transitional B cells (R=0.5857, respectively, p=0.0218). Similar observations were found between the ratios of CD19⁺CD38⁺CD24⁺ mature-naive B and CD4⁺CXCR5⁺PD-1⁺IL-21⁺ T_{FH} cells in pSS (R=0.5536, respectively, p=0.0323). We found a significant positive correlations between the aforementioned cell subsets (transitional B vs. T_{FH}-like cells: R=0.5964, respectively, p=0.0189). A significant negative correlation was observed between the percentages of CD19⁺IgD⁺CD27⁺ non-switched memory B cells and T_{FH} cells (R=-0,6553, respectively, p<0,0001) as well as between the percentages of CD19⁺IgD⁻CD27⁺ switched memory B cells and T_{FH} cells (R=-0,5876, respectively, p<0,0001).

IV.1.11. Association of peripheral T_{FH}-like cells with serological parameters in pSS

We investigated the association of autoantibody titers with the proportions of T_{FH} cells in pSS patients. We found significant positive correlation between the levels of anti-Ro/SSA antibody and the percentages of T_{FH} cells ($R=0.5143$, respectively, $p=0.0243$), as well as between the levels of anti-La/SSB antibody and the proportions of T_{FH} cells ($R=0.6545$, respectively, $p=0.0336$) in the antibody-positive group. When we analyzed the whole patient population we found a significant positive correlation between the ratios of T_{FH}-like cells and serum level of IgG ($R=0.3674$, respectively, $p=0.0016$) and ICs ($R=0.3830$, respectively, $p=0.0036$). A strong association was measured between the percentages of IL-21 producing T_{FH}-like cells and serum levels of ICs ($R=0.5734$, respectively, $p=0.0128$) as well. Moreover, we found a positive correlation between the ratios of IL-21 producing T_{FH}-like cells and the titer of RFs ($R=0.6219$, respectively, $p=0.0101$).

IV.2. Histological results in pSS

IV.2.1. Systemic characteristics of the study population during the course of the disease

We evaluated their clinical and serological features retrospectively, and assessed the relation between laboratory results, disease course and the early histopathological findings. Peripheral T_{FH}-like cell percentages were tendentially elevated at the end of follow-up in patients with higher FS at disease onset. Importantly, systemic features such as polyarthritis ($n=3$), Raynaud's syndrome ($n=2$), lymphadenopathia ($n=1$) and fibrosis pulmonum ($n=1$), and associated diseases including primary biliary cirrhosis ($n=1$) or primary sclerosing cholangitis ($n=1$) developed later in the disease course only in patients with FS=3.

IV.2.2. Histological classification of LSG biopsies according to focus scoring and grading the inflammatory infiltrates

When studying the morphology of LSG specimens in patients with pSS, we identified different organizational levels of inflammatory mononuclear cell infiltrates. The whole LSG specimen was characterized based on the FS, while the extension and the structural arrangement level of each periductal cellular infiltrate was graded within the biopsy section. In our study, the biopsy samples with FS=2 consisted of lymphocytic aggregates only graded as 1 or 2. More organized follicles as grade 3 or 4 were exclusively found in pSS with FS=3. Grade 4 lymphocytic foci exhibited features of GCs within secondary lymphoid organs.

IV.2.3. Immunohistochemical characterization of infiltrating cells according to the cell-specific markers in LSG biopsies

In the aggregates of pSS group with FS=2, mainly the T helper cell marker CD4, CD5 and the pan-B cell marker CD20 were detected, while the T_{FH}-related markers CD84 and PD-1 were less evident. Cells characterized by the above mentioned molecules showed scattered distribution within the infiltrates. In pSS group with FS=3, the distribution of specific cell markers showed a different pattern along with more organized structures. CD4⁺ T cells were predominantly localized at the periphery of infiltrates. The CD20⁺ B cells were principally situated at the central region of lymphoid follicles. Similarly to pSS group with FS=2, CD138⁺ plasma cells were also displayed a scattered distribution outside the infiltrates, however some of them were observed at the border of B cell zone as well. The expression of CD84 cell surface molecule was diffused throughout the inflammatory infiltrate but accumulated at the inner area. In addition, the expression of PD-1 was solely found in the location of CD20⁺ B cells. Bcl-6⁺ cells were detected exclusively in pSS with FS=3. After analysing the pSS group with FS=3, intragroup variances were discovered; at grade 4 organization level Bcl-6⁺ cells were clustered in the central region and expressed with higher intensity, while in grade 3 aggregates Bcl-6⁺ cells were scattered and showed much lower expression.

IV.2.4. Double immunofluorescence for the demonstration of T_{FH}-related Bcl-6 with possible T or B cell co-expressions in autoreactive lymphocytes of LSG biopsies

To prove that CD3⁺Bcl-6⁺ T cells were involved in the formation of GC-like structures in LSG, we stained sections by double IF for Bcl-6 and CD3 or CD20 expressions. We showed the double labeling of CD3 pan-T cell marker with the transcription factor Bcl-6 in lesional lymphocytes, indicating that a few T cells in the infiltrates were positive for Bcl-6. The co-expression of the two markers clearly identified T_{FH} cells. Bcl-6⁺ B cells with the typical formation of conventional GCs have also been detected in the central area of the lymphoid follicle.

V. DISCUSSION

The immunopathogenesis of pSS and SLE are highly complex and are associated with both the adaptive and innate branches of the immune system. Besides the presence of specific serological features, patients are also characterised by fundamental disturbances in the

frequency of different B cell subpopulations, both in the peripheral blood as well as in the site of the inflammation. Physiologically, to avoid autoreactivity, during B cell development multiple tolerance checkpoints are applied for the selection of autoreactive B cells. Nevertheless, abnormalities in checkpoint regulation or the hyperactivation of T_{FH} cells and self-reactive B cells contribute to the development of autoimmunity in susceptible individuals as demonstrated by our work, revealing the importance of the accumulation of circulating T_{FH}-like cells and aberrant distribution of B cell subsets at the periphery.

Patients with pSS and SLE are characterised by fundamental disturbances in the proportion of different B cell subpopulations, both in the peripheral blood and the site of inflammation. In our study, we found a significant enrichment of CD19⁺IgD⁺CD27⁻ naive B cells in the peripheral blood of both pSS and SLE patients compared to healthy individuals. Our observations indicate that early B cell tolerance checkpoints are significantly impaired in these autoimmune diseases; moreover the break of tolerogenic mechanism of this stage probably accelerates the mobilization of autoreactive naive B cells from the bone marrow to the periphery. There is another major tolerance checkpoint during the maturation stage of immature B cells when transitional B cells overcome a negative selection. In accordance with previous findings, we observed significant elevation in the percentages of transitional B cells in SLE patients, additionally, this cell population showed association with the disease activity. In pSS, the frequency of transitional B cells did not correlate with the presence of EGMs. However, when we divided pSS patients into subgroups based on the presence of anti-SSA/Ro autoantibodies, we observed significantly higher transitional B cell proportions in pSS patients with autoantibody positivity, and found a positive association between elevated cell ratios and serum IgG levels. The pathologic accumulation of these cells may occur due to their increased exiting from the bone marrow or disturbed entrance into secondary lymphoid organs. Of note, the defect in early self-tolerance may also cause the expansion of circulating self-reactive and polyreactive type of mature-naïve B cell subset. In our study, we measured significantly higher percentages of CD19⁺CD38⁺CD24⁺ mature-naïve B cells in SLE. Importantly, large numbers of autoreactive B cells occur among the mature-naïve B cell compartment in SLE. We also confirmed that peripheral CD19⁺IgD⁺CD27⁺ non-switched memory B cells and CD19⁺IgD⁻CD27⁺ switched memory B cells are strongly diminished in both pSS and SLE. Additionally, we revealed significant differences between the distributions of the two memory B cell compartments in the investigated diseases. In pSS patients, the proportion of switched memory B cells decreased significantly; while in SLE patients, the non-switched memory B cells reduced significantly. Furthermore, within pSS and SLE patient

groups, a more pronounced reduction was observed in patients with EGMs or higher SLEDAI values. In addition, among SLE patients, individuals with active disease status exhibited a significant decrease in switched memory B cell subset, which underlines the importance of the changing distribution of B cell subsets during the disease course. The lower ratio of circulating memory B cells may be explained by their overexpression of chemokine molecules CXCR3 and CXCR4 which guide them into the inflamed tissues. We detected significantly increased CD19⁺IgD⁻CD27⁻ DN B cells in SLE patients, while the ratio of DN B cells was significantly decreased in pSS patients. However, regarding the clinical features, these differences were found only in patients with a more pronounced disease course, such as high SLEDAI or the presence of EGMs. A previous report proposed that the presence of these cells could be the result of an extrafollicular differentiation process in secondary lymphoid organs. The expression of CD95 indicates that they are in an activated state; however their activation does not require T cell interaction. Furthermore, due to the expression of CXCR3, after receiving activation signals, DN B cells could migrate to inflamed tissues. The proportion of CD19⁺CD38^{hi}CD27^{hi} plasmablasts were significantly decreased in pSS patients with EGMs, while their ratio was significantly elevated in SLE patients with SLEDAI>6. This discrepancy may arise from the heightened expression of CXCR3 and CXCR4 on plasma cells and plasmablast of patients with pSS, which leads to their migration toward the site of inflammation or even the bone marrow. Moreover, in SLE, the differentiation toward plasmablasts is more pronounced, compared to pSS, and could be originated from both T-dependent and T-independent responses. In the present study, we analysed the whole population of IL-10 producing B cells (B10 and B10_{PRO}) upon with the activation with CpG-ODN. Originally, Breg cells play an important role in maintaining the homeostasis of the immune system. They form a heterogeneous cell population, but they can identify based on their ability to produce IL-10. We observed the expansion of IL-10 producing B cells during the activation; however the patient's data are remained lower than controls, especially in the CD19⁺CD38⁺CD24⁺ B cell population.

T_{FH} cells are crucial immune regulators in secondary lymphoid follicles by controlling B cell proliferation and differentiation. Since the measurement of human GC T_{FH} cell from lymphoid follicles could be hardly performed, the investigation of circulating T_{FH}-like cells are widely accepted. In the present study, we revealed a significant increase in the proportion of peripheral CD4⁺CXCR5⁺ICOS⁺PD-1⁺ T_{FH}-like cells in pSS patients with EGMs compared to control values, while values of pSS patients without EGMs were similar to healthy individuals. Moreover, we also observed a significant difference between patients with and

without EGMs. When we divided patients into subgroups based on the presence of anti-SSA/Ro, we found significantly higher T_{FH}-like cell percentages in autoantibody positive group. The same changes were measured in SLE, and circulating T_{FH}-like cell percentages were significantly elevated in patients positive for anti-dsDNA, however there was no significant difference between the inactive and active status of the disease. 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. 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_{FH} 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_{FH} cells. As next step, we evaluated the proportion of IL-21 producing CD4⁺CXCR5⁺PD-1⁺ T_{FH}-like cells and found a significant elevation in pSS patients with EGMs and/or anti-SSA/Ro positivity. Similarly, a marked expansion was observed in SLE patients, which was independent from the disease status, however the tendency was more pronounced in patients with anti-dsDNA autoantibody. 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. Therefore we studied levels of soluble IL-12 cytokine in sera of pSS patients. According to our results, IL-12 positive patients had significantly higher percentages of T_{FH} cells. During our work, by T_{FH}-like cells, we extensively investigated the changes of the immune competent cells and laboratory parameters as well. In the international literature, we provided the first complex data about the role of these cells in pSS. In our present study, we found positive correlations between the percentages of T_{FH} cells and early- and late-activated T cells, which indicate that in parallel with the activation of immune system, a stronger T_{FH} cell expansion can be observed. T_{FH} 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. Regarding B cell subsets, the frequency of transitional B cells correlated with the increased T_{FH}-like cell and IL-21⁺ T_{FH}-like cell percentages in pSS; furthermore, the level of mature-naive B cells also correlated with the increased ratio of T_{FH}-like cells. These observations suggest that disturbances in early

B cell tolerance mechanisms may lead to the peripheral expansion and further accumulation of these B cell subsets. B cell hyperreactivity, development of autoantibodies, and disturbance in distribution of B cell subtypes on the periphery are characteristics in pSS. Our results support partially the aforementioned observations, since we found a negative correlation between the proportions of T_{FH} cells and non-switched and switched memory B cells in the peripheral blood. These can be explained by the enhanced differentiation toward plasma cells or the heightened migration into inflammatory tissues of the memory B cells. To get a better view on the significance of T_{FH}-like cell expansion in pSS, we analysed the association between the proportion of T_{FH}-like cells and other parameters. Our results revealed a positive correlation between circulating T_{FH}-like cells and serum levels of IgG and RF. These parameters are strongly related to the regulation of plasma cell generation which underlines the role of T_{FH} cells in autoimmune diseases. Obtaining LSG biopsy is part of the routine diagnosis procedures in pSS provides an excellent opportunity to reveal the severity of autoimmune inflammatory processes in the early stage of the disease. Before our present investigations, T_{FH} cells were not studied in glandular lymphocytic infiltrates with different organizational levels. In our study, we classified LSG specimens according to the severity of inflammatory cell infiltrates not only with focus scoring but also with grading of the lymphoid aggregates. To determine the FS and the grades of aggregates we examined the entire tissue section. We observed that the biopsy samples contained different grades of mononuclear cell infiltrates, and the periductal lymphoid structures showed higher level of organization in pSS group with FS=3 than pSS group with FS=2. Ectopic GC structures with peripheral positioned T cells, centrally localized B cell area and a reticular pattern of FDC network were only observed in FS=3 with grade 4 aggregates. When examining the expression of T_{FH}-related molecules such as CD84, PD-1 and Bcl-6 in the infiltrates, we found a pronounced expression in pSS with FS=3. Marked Bcl-6 expression was detected only in grade 4 aggregates with the co-localization of B cell zone. It is known, that Bcl-6 is specially expressed by GC B cells during the centroblast phase and usually, but not consistently, in centrocytes as well. According to experimental studies, *BCL6* gene defect resulted in disturbed GCs formation with the lack of SHM and CSR, which highlights the role of Bcl-6 in GC responses. For that purpose, we paid a special attention to the presence and localization of T_{FH} and GC B cells in the mononuclear cell infiltration. With double IF staining, we demonstrated that close to B cell area, a certain subset of infiltrating T cells expressed both CD3 and Bcl-6 markers, which suggests that the presence of T_{FH} cells was adjacent to GC B cells in LSG lesions. It is important to emphasize that our investigations were performed on LSG biopsies which were

collected at the time of the diagnosis, when only the initial symptoms developed in patients. The retrospective evaluation of both laboratory and clinical data recorded during the follow-up period, revealed associations between the formation of GCs with the presence of T_{FH} cells in LSGs at disease onset and the development of EGMs and associated diseases during the disease course. Nevertheless, the present findings are in line with our earlier observations, that the higher proportions of T_{FH} cells are associated with higher FS in glandular biopsies and the presence of extraglandular manifestations. In summary, we can state that we could shed light on the pathological role of T_{FH} cells in autoimmune processes which contribute to the development of the characteristic features, namely the abnormal B cell responses and diagnostic parameters in both pSS and SLE. The understanding of the immunobiology of T_{FH} cells is in the focus of today's research and we hope that further studies on their development and function can provide further valuable information in order to serve as potential therapeutic targets in the treatment of autoimmune diseases.

VI. SUMMARY

The immunopathogenesis of pSS and SLE are highly complex and are associated with both the adaptive and innate branches of the immune system. Pronounced B-cell hyperactivity appears to be the hallmark of diseases. T_{FH} cells have a crucial role in regulating immune responses within secondary lymphoid follicles by directing B cell differentiation toward memory B cells and plasma cells. Since abnormal humoral responses are key features in both diseases, the aim of this study was to profile the pathological connection between peripheral T_{FH} cells and B cells. We observed higher percentages of naive B cells in both diseases, while non-switched and switched memory B cells showed decreased frequencies. The proportions of DN B cells and plasmablasts were elevated in SLE and decreased in pSS. The percentages of transitional B cells and mature-naive B cells were higher in SLE. Patients with more severe disease course had elevated ratio of T_{FH}-like cells and increased IL-21 production. Moreover, expansion of T_{FH}-like cells correlated positively with parameters related to antibody secretion, including serum IgG, ICs and autoantibodies. Our observations on the profound expansion of circulating T_{FH}-like cells and their IL-21 production along with the characteristic aberrant peripheral B cell distribution in both pSS and SLE indicate the prominent role of T_{FH} cell in the regulation of B cell selection. Furthermore, we examined the composition of lymphocyte infiltration in labial salivary gland (LSG) biopsies from patients with pSS, focusing on the presence of B and T_{FH} cells. T_{FH} cell markers are predominantly occurred in more organized

lymphocyte infiltrates with higher focus scores. Interestingly, upon assessing the clinical data of patients retrospectively, we found association between the presence of T_{FH} cells in LSGs at onset and the severity of clinical course. Our results suggest that T_{FH} cells play important role in the generation of autoreactive B cells in glandular infiltrations and may contribute to the subsequent systemic manifestations as well.

VII. PRESENTATIONS AND POSTERS ARE BASED ON THE THESIS

- **Szabó Krisztina**

DE OEC, ÁOK, Belgyógyászati Intézet, Klinikai Immunológiai Tanszék

A follikuláris T helper sejtek pathológiás szerepe az immun-diszregulációban a Sjögren-szindróma különböző fenotípusai esetén. (KIADI III. PhD Konferencia, 2011. 11. 25.)

- **Szabó Krisztina, Papp Gábor, Baráth Sándor, Szántó Antónia, Zeher Margit**

DE OEC, ÁOK, Belgyógyászati Intézet, Klinikai Immunológiai Tanszék

A follikuláris T helper sejtek pathológiás szerepe az immun-diszregulációban a Sjögren-szindróma különböző fenotípusai esetén. (MAKIT 40., 2012. május 17-19. Balatonalmádi, Immunológia-Allergológia Szekció) – *Legjobb előadás díja*

- **Szabó Krisztina**

DE OEC, ÁOK, Belgyógyászati Intézet, Klinikai Immunológiai Tanszék

A periférián található follikuláris T helper sejtek patológiás szerepe primer Sjögren-szindrómában. (KIADI IV. PhD Konferencia, 2012. 09. 28.)

- **Szabó Krisztina, Papp Gábor, Baráth Sándor, Gyimesi Edit, Szántó Antónia, Zeher Margit**

DE OEC, ÁOK, Belgyógyászati Intézet, Klinikai Immunológiai Tanszék

A periférián található follikuláris T helper sejtek patológiás szerepe primer Sjögren-szindróma különböző fenotípusai esetén. (MIT 41., 2012. október 17-19. Debrecen, Kölcsey Központ, IV. szekció)

- **Krisztina Szabó, Gábor Papp, Margit Zeher**

Division of Clinical Immunology, Institute of Medicine, University of Debrecen

Increased proportions of peripheral follicular helper T cells in patients with primary Sjögren's syndrome. (EMESCO 2012. Oral Session V - Immunology, Microbiology)

- **Krisztina Szabo, Gabor Papp, Sandor Barath, Edit Gyimesi, Antonia Szanto, Margit Zeher**

Division of Clinical Immunology, Institute of Medicine, University of Debrecen

Investigation of peripheral follicular helper T cells in primary Sjögren's syndrome. (WISC 2012. Hyderabad, India, 6-9 December, Poster Session 3-3: Basic and Clinical Immunology 2, 3036)

- **Krisztina Szabo, Gabor Papp, Sandor Barath, Edit Gyimesi, Antonia Szanto, Margit Zeher**

Division of Clinical Immunology, Institute of Medicine, University of Debrecen

Peripheral follicular helper T cells correlate with B cell activation and autoantibody production in primary Sjögren's syndrome. (CORA 2013, Budapest, Hungary, 4-6 April, Poster Session/Walk 1: Tissue issues, P91, Thursday, April 4, 2013)

- **Szabó Krisztina, Papp Gábor, Szegedi Andrea, Zeher Margit**

DE OEC, ÁOK, Belgyógyászati Intézet, Klinikai Immunológiai Tanszék

A follikuláris helper T sejtek kiemelt szerepet játszhatnak bizonyos autoimmun és allergiás betegségek pathogenezisében. (MAKIT 41., 2013. május 9-11. Balatonalmádi, Immunológia Szekció)

- **Krisztina Szabo, Gabor Papp, Sandor Barath, Margit Zeher**
Division of Clinical Immunology, Institute of Medicine, MHSC, University of Debrecen
Follicular helper T cells may play an essential role in the severity of certain autoimmune disorders. (8th ENII, Porto Conte, Sardinia, Italy, 27 May-3 June, 2013, Poster Session D: P7, Friday, May 31, 2013)
- **Szabó Krisztina**
DE OEC, ÁOK, Belgyógyászati Intézet, Klinikai Immunológiai Tanszék
A folliculáris T helper sejtek pathológiás szerepe az immun-diszregulációban a Sjögren-szindróma különböző fenotípusai esetén. (TAIM Nemzetközi Alapítvány XI. Szakmai Továbbképzése, 2013. augusztus. 23-24. Debrecen) - *Pályadíjasok előadása, I. helyezés.*
- **Szabó Krisztina**
DE OEC, ÁOK, Belgyógyászati Intézet, Klinikai Immunológiai Tanszék
A folliculáris T helper sejtek pathológiás szerepének immunhisztokémiai vizsgálata primer Sjögren-szindrómás betegek kisnyálmirigy biopsziás mintáiban. (KIADI V. PhD Konferencia, 2013. október 4. Debrecen)
- **Szabó Krisztina, Papp Gábor, Szántó Antónia, Tarr Tünde, Zeher Margit**
DE ÁOK, Klinikai Immunológiai Tanszék
Folliculáris T helper sejtek és IL-10 termelő regulatív B sejtek vizsgálata primer Sjögren-szindrómában és szisztémás lupus erythematosusban. (MAKIT 43., 2015. május 7-9. Kecskemét, Poszter szekció II.)
- **K. Szabó¹, G. Papp¹, S. Baráth¹, E. Gyimesi¹, B. Dezső², A. Szántó¹, M. Zeher¹**
¹Division of Clinical Immunology, Faculty of Medicine, University of Debrecen
²Department of Pathology, Faculty of Medicine, University of Debrecen
The role of follicular T helper cells in altered B cell functions in primary Sjögren's syndrome. (ISSS 2015, Norway, Bergen, 19-22 May, 2015, Poster Session S7:12)
- **Szabó Krisztina, Papp Gábor, Tarr Tünde, Szántó Antónia, Zeher Margit**
DE ÁOK, Klinikai Immunológiai Tanszék
Cirkuláló folliculáris T helper sejtek és B sejt alcsoportok vizsgálata autoimmun kórképekben. (TAIM Nemzetközi Alapítvány XII. Szakmai Továbbképzése, 2015. augusztus 27-28. Debrecen)
- **Szabó Krisztina, Papp Gábor, Szántó Antónia, Tarr Tünde, Zeher Margit**
DE ÁOK, Klinikai Immunológiai Tanszék
A folliculáris T helper sejtek és B sejt alcsoportok megoszlásának átfogó vizsgálata primer Sjögren-szindrómában és szisztémás lupus erythematosusban. (MRE 2015, 2015. szeptember 24-27. Szeged, „legjobb előadások díja” jelöltjeinek előadásai)

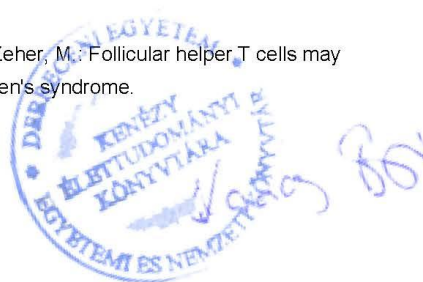


Registry number: DEENK/15/2016.PL
Subject: Ph.D. List of Publications

Candidate: Krisztina Szabó
Neptun ID: GJX1AG
Doctoral School: Doctoral School of Clinical Immunology and Allergology
MTMT ID: 10043545

List of publications related to the dissertation

1. Papp, G., Gyimesi, E., **Szabó, K.**, Zöld, É., Zeher, M.: Increased IL-21 Expression Induces Granzyme B in Peripheral CD5+ B Cells as a Potential Counter-Regulatory Effect in Primary Sjögren's Syndrome.
Mediat. Inflamm. 2016, 1-8, 2016.
DOI: <http://dx.doi.org/10.1155/2016/4328372>
IF:3.236 (2014)
2. **Szabó, K.**, Papp, G., Szántó, A., Tarr, T., Zeher, M.: A comprehensive investigation on the distribution of circulating follicular T helper cells and B cell subsets in primary Sjögren's syndrome and systemic lupus erythematosus.
Clin. Exp. Immunol. Epub, 2015.
DOI: <http://dx.doi.org/10.1111/cei.12703>
IF:3.037 (2014)
3. **Szabó, K.**, Papp, G., Dezső, B., Zeher, M.: The histopathology of labial salivary glands in primary Sjögren's syndrome: Focusing on follicular helper T cells in the inflammatory infiltrates.
Mediat. Inflamm. 2014, 11 p., 2014.
DOI: <http://dx.doi.org/10.1155/2014/631787>
IF:3.236
4. **Szabó, K.**, Papp, G., Baráth, S., Gyimesi, E., Szántó, A., Zeher, M.: Follicular helper T cells may play an important role in the severity of primary Sjögren's syndrome.
Clin. Immunol. 147 (2), 95-104, 2013.
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List of other publications

5. Csillag, A., Kumar, B.V., **Szabó, K.**, Szilasi, M., Papp, Z., Szilasi, M.E., Pázmándi, K., Boldogh, I., Rajnavölgyi, É., Bácsi, A., László, F.J.: Exposure to inhomogeneous static magnetic field beneficially affects allergic inflammation in a murine model.
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DOI: <http://dx.doi.org/10.1098/rsif.2014.0097>
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6. **Szabó K.**, Zeher M.: A follicularis T-helper-sejtek jellegzetességei és lehetséges szerepük az autoimmun és immunhiányos kórképekben.
Immunol. Szle. 5 (3), 48-55, 2013.
7. Pázmándi, K., Kumar, B.V., **Szabó, K.**, Boldogh, I., Szőör, Á., Vereb, G., Veres, Á., Lányi, Á., Rajnavölgyi, É., Bácsi, A.: Ragweed subpollen particles of respirable size activate human dendritic cells.
PLoS One. 7 (12), e52085, 2012.
DOI: <http://dx.doi.org/10.1371/journal.pone.0052085>
IF:3.73

Total IF of journals (all publications): 21,148

Total IF of journals (publications related to the dissertation): 13,501

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

21 January, 2016

