Differential Th follicular cell subsets in minor salivary glands of patients with primary Sjögren's syndrome and systemic lupus erythematosus associated with Sjögren's syndrome

G. Hernandez-Molina[1], V. Soto-Abraham[2], V. Zamora-Legoff[1], J. Furuzawa-Carballeda[1]

[1]Department of Immunology and Rheumatology, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Mexico City; [2]Department of Pathology, Instituto Nacional de Cardiología Ignacio Chávez Mexico City, Mexico.

Gabriela Hernandez-Molina, MS
Virgilia Soto-Abraham, MD
Victor Zamora-Legoff, MD
Janette Furuzawa-Carballeda, PhD

Please address correspondence to:
Janette Furuzawa-Carballeda,
Department of Immunology and Rheumatology,
Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubiran,
Vasco de Quiroga 15,
Colonia Belisario Domínguez,
Sección XVI,
CP 14080 Mexico City, Mexico.
E-mail: jfuruzawa@gmail.com

Received on July 3, 2021; accepted in revised form on September 15, 2021.

Clin Exp Rheumatol 2021; 39 (Suppl. 133): S49-S56.

Key words: follicular T cells, Sjögren's syndrome, systemic lupus erythematosus, Tfr cells

Competing interests: none declared.

ABSTRACT

Objective. *T follicular helper cells (Tfh) have been recognised in minor salivary glands (MSG) of patients with primary Sjögren's syndrome (pSS). Nevertheless, if the Tfh1, Tfh2, Tfh17, Tfr phenotype is different when comparing pSS and associated SS in systemic lupus erythematosus (SLE) is unknown.*

Methods. *We included MSG biopsies from 8 pSS, 8 SLE/SS patients, 7 SLE patients, and 2 non-SS sicca patients. To determine the subpopulation of Tfh, a double-staining procedure for transcription factor B cell lymphoma 6 (Bcl-6)+/IL-17A+, Bcl6+/IL-4+, Bcl6+/IFN-γ+, and Bcl6+/Foxp3+ cells was performed. We estimated the mean percentage of positively staining cells in four fields per sample.*

Results. *Tfh1, Tfh2, and Tfh17 cells were highly expressed in pSS compared with the rest of the groups; conversely, in patients with SLE/SS predominated, the Tfh17 and in SLE patients the Tfh1 cells. Regulatory Tfh cells (Tfr) were similar in pSS and the rest of the patients. However, the lowest frequency was found in the SLE group. A positive correlation was observed between anti-Ro/SSA autoantibody and Tfh17 subset ($r=0.726$, $p=0.0001$); and with the (Tfh2+Tfh17)/Tfh1 ratio ($r=0.844$, $p<0.0001$) in the MSG of patients with pSS.*

Conclusion. *We showed a differential Tfh profile in primary SS and SLE with associated SS. Whether this Tfh differential profile participates in the increased risk of lymphoproliferative disease in pSS compared with associated SS, or other outcomes, is yet to be determined in future studies.*

Introduction

Sjögren's syndrome (SS) is a chronic autoimmune disease characterised by focal periductal lymphocytic sialoadenitis, where early infiltrating cells are mainly $CD4^+$ T, followed by B lymphocytes. At the salivary and lachrymal glands, T and activated B cell interactions occur in the germinal centre (GC) like structures (1-2).

T follicular helper (Tfh) cells have been identified as a $CD4^+$ T cell subset generated in the T cell zone of lymphoid organs. The interplay between B cells and Tfh is essential for the formation of GC, regulating B-cell differentiation, and producing high-affinity autoantibodies. The cells highly expressed the CXC chemokine receptor 5 (CXCR5), the inducible T- cell costimulator (ICOS) molecule, the co-inhibitory molecule programmed cell death protein 1 (PD-1), and the transcription factor Bcl6, and typically produce IL-21 (3).

Moreover, depending on the presence of the chemokine receptors, three major subsets are recognised: CXCR3+CCR6- cells that share properties with Th1 cells (express IFN-γ and the transcription factor T-bet), CXCR3-CCR6- resembling Th2 cells (express IL-4, IL-5, IL-13, and the transcription factor Gata3) and CXCR3-CCR6+ cells resembling Th17 cells (express IL-17A, IL-22 and the transcription factor RORγ) (4). The cell subsets can also be divided into non-efficient helpers (Tfh1) and efficient helpers (Tfh2 and Tfh17). Tfh17 cells produce IL-21 and induce B cells proliferation, differentiation into Ab-producing cells, and class-switching to IgG and IgA, whereas Tfh1 cells lack of this capacity (5). Various autoimmune diseases are associated with higher B-helper Tfh subsets (Tfh2 and Tfh17) and/or a decrease in non-B helper Tfh subsets (Tfh1) or regulatory subset (Tfr) (6).

$CD4^+CXCR5^+$Tfh follicular cells have been described within or outside GC in minor salivary glands (MSG) of patients with pSS, as well as in peripheral blood (7-11). Indeed, their

presence in peripheral blood have been associated with extra-glandular features, anti-Ro/SSA positivity (7, 11), a higher EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI) (10, 12), and Clinical EULAR Sjögren's Syndrome Disease Activity Index (ClinESSDAI) scores (12), and a higher focus score (13). Moreover, their depletion is observed after rituximab treatment (14). Also, in ectopic lymphoid structures in salivary glands and MALT parotids, the presence of $CXCR5^+CD4^+PD1^+ICOS^+Foxp3^-$Tfh-cells have been identified (15).

In addition, a specialised type of Treg cells, named Tfr cells, can inhibit GC responses by controlling the number of Tfh and GC B cells (8). In pSS, peripheral $CXCR5^+Foxp3^+$ Tfr cells and the Tfr/Tfh ratio are increased *versus* non-SS sicca controls (12, 16).

On the other hand, SLE and pSS are close chronic inflammatory clinical conditions of autoimmune nature. Tfh cells also contribute to lupus pathogenesis, and an increased frequency has been associated with SLE disease severity, anti-DNA antibodies (17-19), and the presence of nephritis (20).

Herein we analysed the presence of different Tfh cells subsets (Tfh1, Tfh2, Tfh17, and Tfr) in MSG biopsies of patients with pSS, SLE with associated SS (SLE/SS), SLE, and non-SS sicca patients.

Materials and methods

Patients

It was an exploratory, observational, and cross-sectional study that included 8 biopsies of MSG of patients with pSS and 7 MSG biopsies of patients with SLE-SS. We also included 8 MSG biopsies of patients with SLE without SS (controls) and 2 MSG biopsies of patients with non-SS sicca symptoms. The patients attended the Department of Immunology and Rheumatology at the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, a tertiary care centre.

Primary SS patients fulfilled the ACR/EULAR 2016 classification criteria (21), did not meet any other autoimmune disease classification criteria, and did not have concomitant lymphoma. Patients with SLE fulfilled the 1997 classification criteria (22). SLE and SLE/SS patients were drawn from our parent study (23) and were not under induction treatment with high doses of prednisone or/and cyclophosphamide or mycophenolate mofetil. However, patients might be receiving antimalarials, azathioprine, methotrexate, and/or prednisone.

All subjects had a face-to-face interview with one rheumatologist using a standardized form, including demographic data, medications, and symptoms. Then, the participants were subjected to a three sequential phase evaluation: screening [European sicca questionnaire, Schirmer-I test, and wafer test, which is a semi-quantitative test to screen for xerostomia (24)], confirmatory [fluorescein staining test, non-stimulated whole salivary flow, anti-Ro/SSA and anti-La/ SSB antibodies] and lip biopsy (23). The diagnosis of focal lymphocytic sialadenitis was established on H&E based on a focal score of ≥1 lymphocytic foci (>50 lymphocytes per 4 mm2) (21). Those biopsies with not enough material, artifacts, or inappropriate backgrounds were eliminated. For the present study, we only included patients with SS with a focal sialadenitis pattern.

Non-SS sicca patients were characterised by a negative MSGB (<1 focus score) and negative anti-Ro/SSA and anti-La/SSB serology.

Immunohistochemistry

To determine the subpopulations of transcription factor B cell lymphoma 6 (Bcl-6)+/IL-17A+, Bcl6+/IL-4+, Bcl6+/IFN-γ+, and Bcl6+/Foxp3+ cells, 4-μm-thick sections of formalin-fixed, paraffin-embedded tissue were placed on positively charged slides. Before staining, the sections were deparaffinised with xylene and rehydrated with a graded series of ethanol and water washes. Following standard dewaxing and rehydration, enzyme antigen retrieval was performed (Enzo Life Sciences, Inc., Farmingdale, NY, USA). The tissue samples were blocked with a peroxidase solution. Then, nonspecific background staining was prevented with an IHC background blocker (Enzo Life Sciences). A simultaneous detection was performed (MultiView (mouse-HRP/rabbit-AP) Enzo Life Sciences). The procedure was a sequential double staining procedure with 10 μg/mL mouse monoclonal/rabbit polyclonal antibodies (Bcl6/IL-17A, Bcl6/IL-4, Bcl6/IFN-γ, and Bcl6/Foxp3; Santa Cruz Biotechnology, CA, USA) were incubated for 30 min at room temperature. The slides were washed and then incubated with the PolyView IHC reagent (mouse-HRP and rabbit-AP) mixture for 20 min. Finally, antigens were visualized using horseradish peroxidase (HRP)/3',3'-diaminobenzidine (DAB), and the second antigen was visualised with alkaline phosphatase (AP)/Permanent Red. The tissue samples were counterstained with Harris haematoxylin and mounted with an aqueous mounting medium. Negative control staining was performed with normal human serum diluted 1:100 instead of a primary antibody and the IHC universal negative control reagent specifically designed to work with rabbit, mouse, and goat antibodies (IHC universal negative control reagent, Enzo Life Sciences). The reactive blank was incubated with phosphate-buffered saline-egg albumin (Sigma-Aldrich) instead of a primary antibody. All controls were used to exclude nonspecific staining or endogenous enzymatic activities. All stained cells were assessed independently of staining intensity by estimating the percentage of positively stained *versus* negatively stained cells in four randomly selected fields (320x). Results were reported as the percentage of immunoreactive cells and expressed as the mean ± standard error of the mean (SEM) of cells quantified by the program IMAGE PRO PLUS version 5.1.1 Media Cybernetics. Silver Spring, MD. USA.

The study was approved by the institutional review board of the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán and was conducted in compliance with the Declaration of Helsinki, the Good Clinical Practice guidelines, and local regulatory requirements. All participants provided written informed consent.

Table I. Clinical and serological characteristics of patients and controls.

	Non-SS sicca group (n=2)	SLE* (n=7)	SLE/SS[&] (n=8)	pSS[#] (n=8)
Demographics				
Age (years)				
Mean ± SD	51.0 ± 7.1	35.0 ± 11.9	37.4 ± 6.9	55.2 ± 14.3
Median (range)	51.0 (46 – 56)	30.3 (25 – 60)	37.2 (28 – 49)	55.5 (34 – 75)
Sex,				
Female %	100	100	100	88
Disease duration (years)				
Mean ± SD	7±2	8.8 ± 6.4	4.6 ± 1.7	1.8 ± 0.9
Median (range)	7.2 (7-7.4)	6.4 (0.7 – 21)	4.8 (1.2 – 6.2)	2.0 (0.6 – 2.8)
Laboratory variables				
Haemoglobin (g/dl))				
Mean ± SD	-	13.3 ± 1.4	10.7 ± 2.6	13.9 ± 1.1
Median (range)		14.1 (10.9 – 14.2)	11.1 (7.2 – 13.4)	13.9 (12.2 – 15.7)
Platelets$^{\wedge}10^{3}$ (cells/µl)				
Mean ± SD	-	249.4 ± 60.3	178.5 ± 21.1	245.6 ± 67.7
Median (range)		224.0 (205 – 353)	173.5 (159 – 208)	235.5 (172 – 377)
Leucocytes$^{\wedge}10^{3}$ (cells/µl)				
Mean ± SD	-	6.3 ± 2.1	6.6 ± 1.3	5.8 ± 3.0
Median (range)		5.5 (4.0 – 9.3)	7.0 (4.9 – 7.7)	4.95 (3.2 – 12.7)
Lymphocytes (%)				
Mean ± SD	-	23.0 ± 10.4	16.9 ± 11.2	32.2 ± 10.6
Median (range)		27.7 (7.4 – 31.7)	16.8 (3.3 – 30.5)	31.6 (18.4 – 50.1)
C3 (UI/ml)				
Mean ± SD	–	119.0 ± 52.9	106.5 ± 27.3	121.7 ± 15.8
Median (range)		123.5 (50 – 179)	117.0 (66 – 126)	124.0 (98 – 141)
C4 (UI/ml)				
Mean ± SD	–	24.5 ± 11.0	28.3 ± 14.9	22.0 ± 6.1
Median (range)		28.5 (8 – 32)	29.5 (13 – 41)	22.0 (12 – 30)
Median anti-Ro/SSA titer U/L (range)	3 (2-4)	3.4 (2.2-5380)	88.4 (26.4-3890)	393 (5.5-2937)
Median anti-La/SSB titer U/L (range)	3 (2-4)	4.4 (2.4-14.6)	16.9 (6-169)	62.8 (3.2-417)
Median rheumatoid factor titer U/L (range)	7.5 (0-15)	9.1 (7.8-42)	12.3 (7.2-449)	28.6 (1.9-231)
Clinical variables				
Oral symptoms, n (%)	2 (100)	6 (85.7)	5 (62.5)	7 (87.0)
Ocular symptoms, n (%)	2 (100)	6 (85.7)	6 (75.0)	7 (87.0)
Schirmer's test, n (%)	1 (50)	3 (42.8)	4 (50.0)	7 (87.0)
Keratoconjuntivitis sicca, n (%)	(0)	2 (28.5)	7 (87.0)	4 (50.0)
Impaired non-stimulated whole salivary flow, n (%)	2 (100)	2 (28.5)	7 (87.0)	5 (62.0)
Treatment				
Prednisone, n(%)	0 (0)	5(71.4)	6(75)	3(37.5)
Antimalarial, n(%)	0 (0)	6(85.7)	4(50)	3(37.5)
Azathioprine, n(%)	0 (0)	4(57.1)	5(62.5)	0 (0)
Methotrexate, n(%)	0 (0)	1(14.2)	2(25)	0 (0)

*SLE: systemic lupus erythematosus; [&]SLE/SS: systemic lupus erythematosus/Sjögren's syndrome; [#]pSS: primary Sjögren's syndrome.

Statistical analysis

A descriptive analysis was done: continuous variables were expressed by means and standard deviations (normal distribution) or medians (non-normal distribution). One-way analysis of variance on ranks by Holm-Sidak and Dunn's methods was performed for all pairwise multiple comparison procedures. Pearson's coefficient of correlation was used for autoantibodies/Tfh subsets. Statistical analysis was done using the SigmaStat11.2 programme (Aspire Software International, Leesburg, VA, USA). Data were expressed as the mean ± standard error of the mean (SEM). The *p*-values smaller than or equal to 0.05 were considered significant. This study conforms to the STROBE statement along with references to STROBE and the broader EQUATOR guidelines.

Results

All the patients were females, except for one patient in the pSS group. The pSS group was the oldest (55 y/o), followed by the SLE/SS group and then the SLE patients. The median disease duration of established diagnosis was 2.4 years for pSS, 4.7 years for SLE/SS, and 6.4 years for SLE. The non-SS sicca group had 7 years of symptoms. The clinical and serological data of the patients and controls are shown in Table I. None of the pSS patients were under immunosuppressors, but 37.5% received prednisone and/or antimalarials. However, in the SLE and SLE/SS groups, around 70% of the patients were under prednisone and immunosuppressors, and at least half of them with antimalarials (Table I).

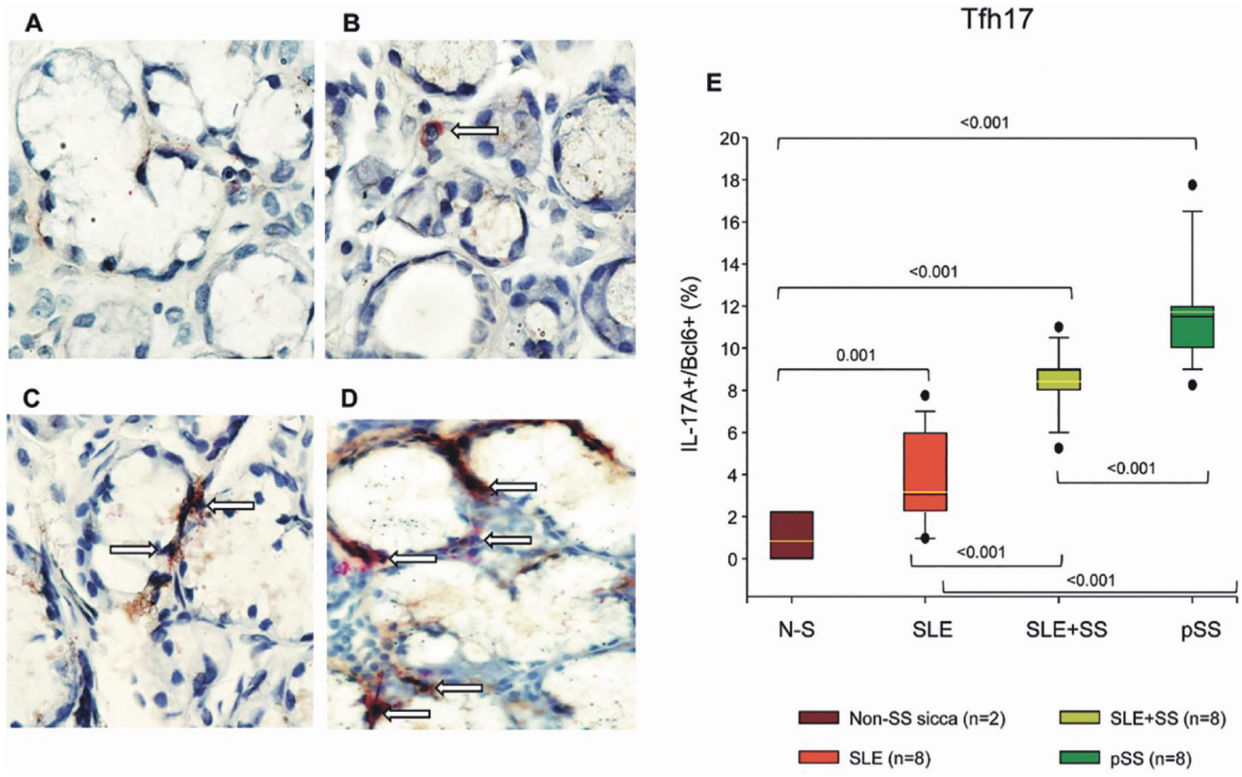

Fig. 1. Tfh17 cells immunostaining in minor salivary gland (MSG) tissue.
A: Non-SS sicca MSG tissue. **B**: Systemic lupus erythematosus (SLE) MSG tissue. **C**: SLE/SS MSG tissue. **D**: Primary SS MSG tissue. Arrows depict Bcl6+/IL-17A+ immunoreactive cells. Original magnification was 320X.
E: Results are expressed as mean (yellow bar), median (black bar), 15th and 95th percentiles.

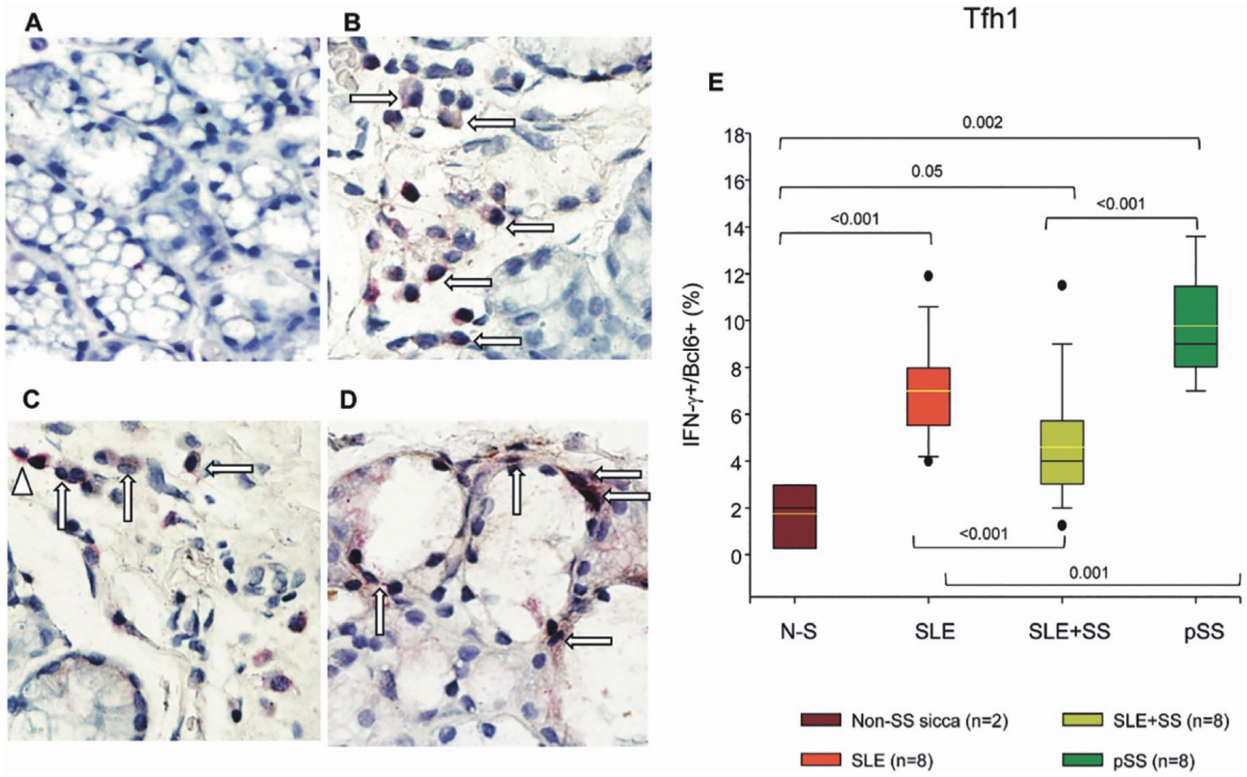

Fig. 2. Tfh1 cells immunostaining in minor salivary gland (MSG) tissue.
A: Non-SS sicca MSG tissue. **B**: Systemic lupus erythematosus (SLE) MSG tissue. **C**: SLE/SS MSG tissue. **D**: Primary SS MSG tissue. Arrows depict Bcl6+/IFN-γ+ immunoreactive cells. Arrowheads show IFN-γ+ cells. Original magnification was 320X.
E: Results are expressed as mean (yellow bar), median (black bar), 15th and 95th percentiles.

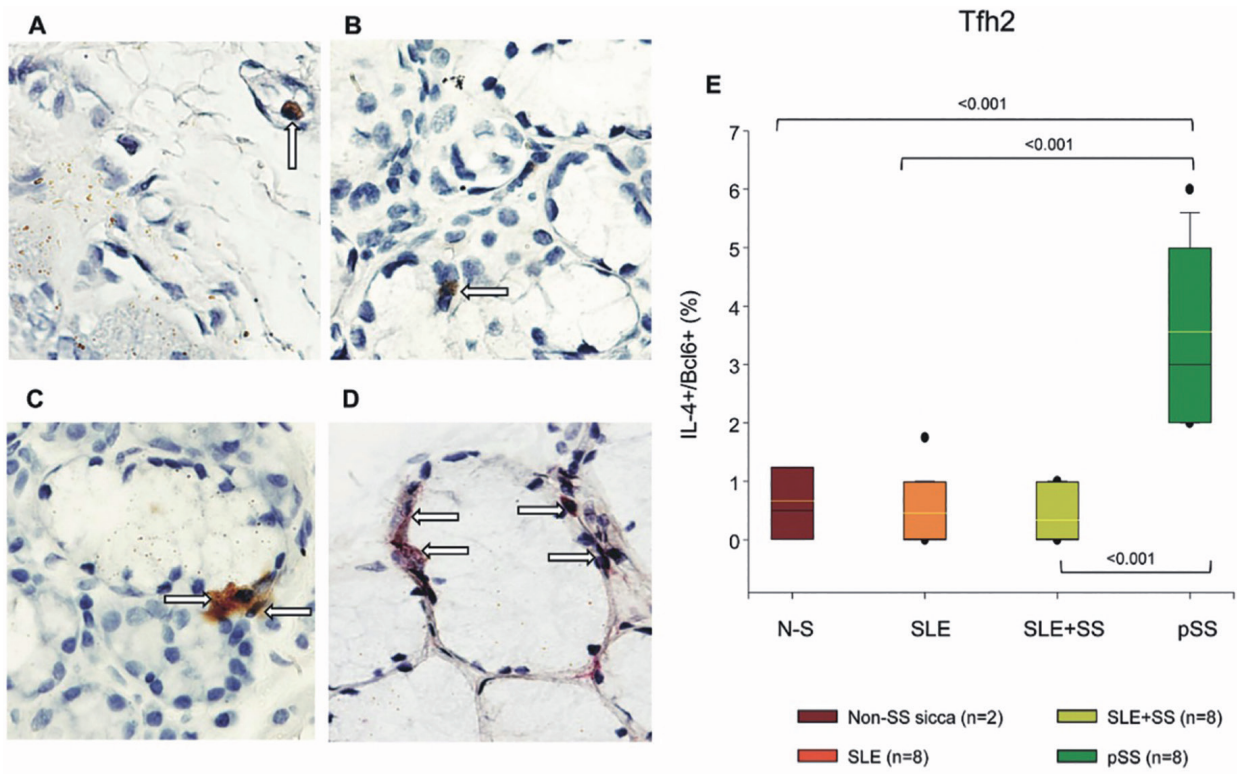

Fig. 3. Tfh2 cells immunostaining in minor salivary gland (MSG) tissue.
A: Non-SS sicca MSG tissue. **B**: Systemic lupus erythematosus (SLE) MSG tissue. **C**: SLE/SS MSG tissue. **D**: Primary SS MSG tissue. Arrows depict Bcl6+/IFN-γ+ immunoreactive cells. Original magnification was 320X.
E: Results are expressed as mean (yellow bar), median (black bar), 15th and 95th percentiles.

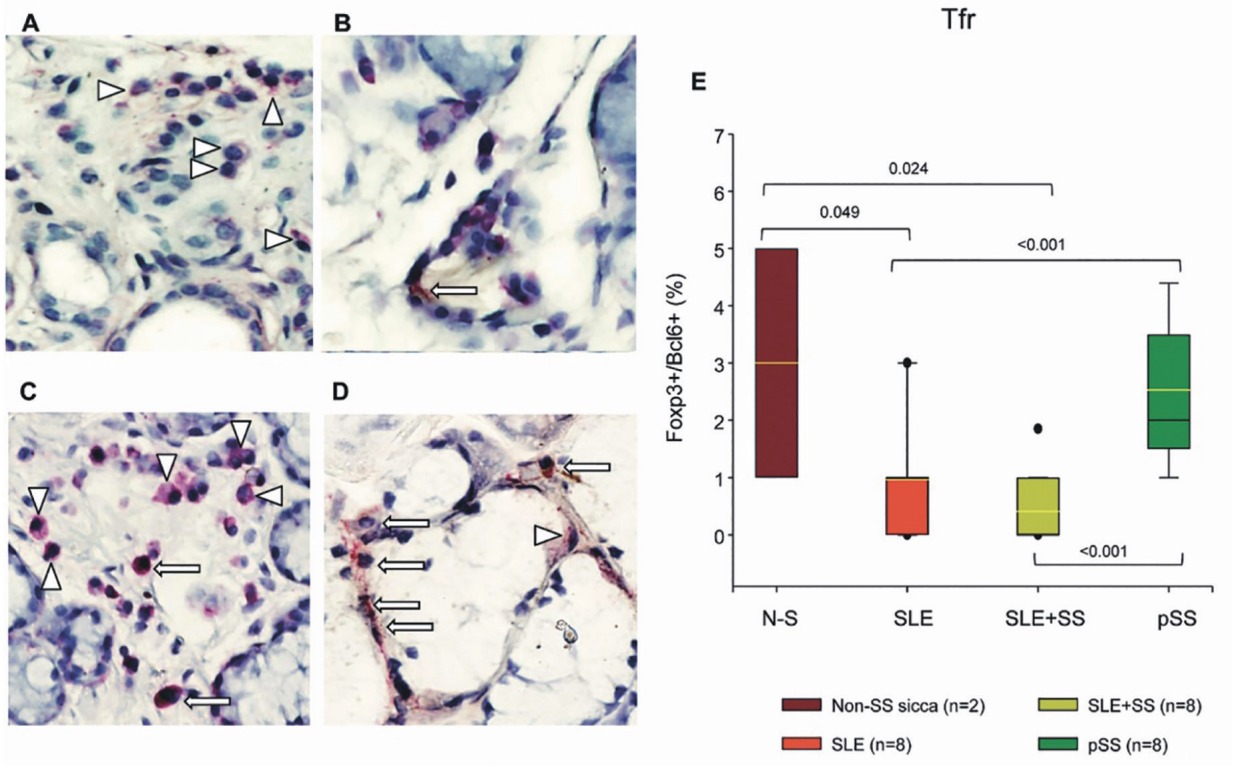

Fig. 4. Tfr cells immunostaining in minor salivary gland (MSG) tissue.
A: Non-SS sicca MSG tissue. **B**: Systemic lupus erythematosus (SLE) MSG tissue. **C**: SLE/SS MSG tissue. **D**: Primary SS MSG tissue. Arrows depict Bcl6+/Foxp3+ immunoreactive cells. Arrowheads show Foxp3+ cells. Original magnification was 320X. **E**: Results are expressed as mean (yellow bar), median (black bar), 15th and 95th percentiles.

Follicular helper T cells

Tfh17 subset. IL-17A-producing Tfh subset was conspicuously higher in MSG of patients with pSS than SLE/SS patients, SLE patients, and non-SS sicca patients. SLE/SS and SLE patients had a higher cell percentage of Tfh17 cells compared to non-SS sicca patients. Finally, SLE/SS patients had more Tfh17 cells than SLE patients (Fig. 1).

Tfh1 subset. IFN-γ-secreting Tfh cells were higher in MSG of patients with pSS than SLE/SS patients and non-SS sicca patients. SLE patients had a higher cell percentage of Tfh1 cells compared to SLE/SS patients and non-SS sicca patients (Fig. 2).

Tfh2 subset. IL-4-producing Tfh cells were higher in MSG of patients with pSS than SLE/SS patients, SLE patients, and non-SS sicca patients. SLE/SS, SLE, and non-SS sicca patients had similar cell percentages of Tfh2 cells (Fig. 3).

Tfr subset. Tfr subset was decreased in MSG of patients with SLE/SS and SLE compared to patients with pSS and non-SS sicca patients. No differences were determined among MSG of patients with pSS and non-SS sicca patients. (Fig. 4).

Correlations among autoantibodies and Tfh subsets

In the pSS group, we observed a positive correlation between anti-Ro/SSA autoantibody and Tfh17 subset in MSG ($r=0.726$, $p=0.0001$). Moreover, anti-Ro/SSA autoantibody was also correlated with the (Tfh2+Tfh17)/Tfh1 ratio ($r= 0.844$, $p<0.0001$). There was not a significant correlation with anti-SSB/La antibody or rheumatoid factor.

We did not find correlations between autoantibodies and the Tfh subsets in the SLE/SS and SLE groups (data not shown).

Discussion

The overlap of SS and SLE, namely associated SS, is well recognised, and its prevalence ranges from around 14%-17.8% (25). Nevertheless, whether or not primary and associated SS are the same entity, is still a matter of debate (26). In addition, both SS and SLE share various clinical, serological, and genetic features, as recently reviewed (27).

Regarding the histological evaluation of salivary glands of SLE/SS patients, Manoussakis *et al*. demonstrated the presence of typical periductal infiltrates as observed in pSS, but also perivascular infiltrates (28). Likewise, other studies confirmed these findings and detected thickening and hyalinization of ductal basement membrane (29-30), epithelial spongiosis with no ductal lymphocytic aggression, vacuolar degeneration of the ductal cells, and serous acinar metaplasia (30). Moreover, by direct immunofluorescence, pSS patients had intercellular ductal IgA deposits, whereas SLE/SS showed deposits of IgG in the ductal basement membrane (29).

Previously, our group reported that based on the immunohistochemistry analysis, pSS patients displayed a significant B lymphocytic infiltration than patients with associated SS (31). Also, when we compared the presence of diverse cytokines and regulatory T and B cells in MSG biopsies among patients with pSS and associated SS, the $CD4^+$/IFN-γ^+, $CD4^+$/IL-4^+, and IL-22+ cell percentages were elevated in both SS varieties; however, these cells were more prevalent in pSS. Patients with pSS also had a high number of $CD4^+$/IL-17A+ and IL-19+ T cells and a lower percentage of IL-24+ cells (32).

$CXCR5^+CD4^+$ cells are specialised memory Th cells primarily involved in GC formation, B cells differentiation, and maturation of high-affinity antibodies. Diverse markers combinations have been used for their identification: 1) the immunoregulatory molecule PD-1 and the chemokine receptor CCR7, 2) the costimulatory molecule ICOS (that indeed define another three subsets, one activated subset (ICOS+PD-12+$CCR7^{lo}$) and two quiescent subsets [ICOS–PD-1+$CCR7^{int}$ and ICOS–PD-1–$CCR7^{hi}$]), and 3) the chemokine receptors CXCR3 and CCR6 (Tfh1, Tfh2, Tfh17) (4,33).

Patients with autoimmune diseases, including pSS and SLE, and display alterations of these phenotypes in peripheral blood. In pSS, some studies have found a higher expression of Tfh2 and Tfh17 subsets (9, 34), but others a higher prevalence of CXCR5+ Th1 and a lower prevalence of CXCR5+Th17 when comparing positive anti-Ro/SSA patients versus the seronegative group (35). A study that included the peripheral blood of patients with SLE, pSS, and controls found a positive correlation between circulating Tfh-cells and IgG and RF serum levels. Also, IL-21 producing Tfh cells showed a positive correlation within serum levels of circulating immunocomplexes, and transitional B cells correlated with the increased Tfh cells and IL-21 (7). Conversely, another work found that the frequency of Tfh17 cells was significantly higher in SLE patients compared to healthy individuals, whereas the frequency of Tfh1 cells was lower (possibly secondary to their migration into inflamed organs due to CXCR3 expression). Tfh1 and Tfh2 cell subsets (but not Tfh17) had been associated with active disease, and also Tfh2 cells with the increase of memory B cells expressing the IL-21 receptor (IL-21R) and high IgE levels in active patients (36).

$CD4^+CXCR5^+$Tfh cells are also significantly increased in MSG biopsies of patients with pSS compared with healthy controls (37). Maehara *et al*. showed expression of $CD4^+CXCR5^+$Tfh IFN-γ and IL-17 in/around the ductal epithelial cells, IL-4 in the acinar cells, and in/around the ectopic GC, and Bcl-6/IL-21 in/around the ectopic GC (38). In the present study, we also observed a higher percentage of Tfh1, Tfh2, and Tfh17 cells in MSG biopsies of pSS. On the other hand, in patients with SLE/SS predominated the Tfh17 cells, and in SLE, we observed a higher prevalence of Tfh1 cells. Nevertheless, the Tfh2 population was similar between SLE patients regarding their SS status. Considering that, Tfh2 cells induce naïve B cells to differentiate into plasmablasts, and they have been characterised as potent inducers of IgG1 class switch recombination for human B cells, we can suggest that in pSS, Tfh2 cells play a crucial role in the pathophysiology of the gland. In contrast, for other diseases, they play a secondary role.

On the other hand, $CD4^+CXCR5^+$Tfh cells have been associated with lymphocytic infiltration (15,34) and the presence of ectopic GC (IL-21+Tfh) (15).

In contrast, Aqrawi *et al*. did not corroborate those associations (39). In that previous study, the authors also reported a lack of correlation with anti-Ro/SSA and the overall $CD4^+CXCR5^+$Tfh cells (39). Nevertheless, in the present study, we observed a positive correlation of anti-Ro/SSA antibody with the Tfh17 subset and the (Tfh2+Tfh17)/Tfh1 ratio, but only in the group of pSS. We might hypothesize that the lack of correlation in the SLE groups might be due to a different anti-Ro52 and anti-Ro60 expression.

Finally, Tfr cells are a subset of $CD4^+CXCR5^+$ Tfh cells also described in GC of MSG biopsies of pSS patients (9, 12, 16, 38). Tfr cells inhibit the differentiation of B-cell into antibody-producing cells and GC formation. Furthermore, in one study of pSS, the blood Tfr/Tfh ratio positively correlated with tissue infiltration by $CD4^+$ T cells and the PD-1+ICOS+ T cells with CD19+ B cells (16); whereas another did not corroborate this finding (12). In SLE, the frequency of circulating Tfr cell is decreased, and the Tfh/Tfr ratio increased (40). Data regarding these population cells in MSG biopsy in associated SS or SLE is not known.

Unlike previous studies, we observed a similar percentage of Tfr cells in the MSG biopsies of patients with pSS compared with non-SS sicca patients. However, SLE patients had the lowest frequency of this cell population.

We acknowledge the limitations of our work. First, the sample was small, and we could not evaluate disease activity and the severity of the lesions. Second, using immunosuppressors and/or prednisone, mainly in the SLE group, might hamper our results. In this sense, a previous study showed that the percentage of peripheral $CD4^+CXCR5^+$Tfh cells was reduced in pSS patients after treatment with glucocorticoid and/or immunosuppressors (37); however, it is widely recognised that steroids do not affect the natural history of chronic sialadenitis in SS. Third, the time of the biopsy was not homogenous, being earlier in the pSS group. If this time difference might hamper our findings, it is uncertain. Nevertheless, in a study that evaluated 28 patients with pSS with repetitive lip biopsies (median rebiopsy time of 55 months), the presence of T cells, Tregs, B cells, macrophages, dendritic cells, and NK cells remained quite similar (41). Finally, we do not know if our results might have the same behaviour at peripheral blood, which is an issue for further research.

Nevertheless, our study has strengths. We used a well-structured approach to establish the SS status in our patients, and the inclusion of patients with a relatively recent diagnosis allowed us to detect the early participation of CD4+CXCR5+Tfh in both primary and associated SS.

Summing up, we showed a differential Tfh profile in pSS and SLE with associated SS. The cells (Th1, Th2, Th17, Tfr) were remarkably present in MSG biopsies of patients with pSS compared to SLE and associated SS; however, the Tfr cell population was similar to non-SS sicca patients. In contrast, associated SS in the context of SLE was driven by Th17 cells. Whether this differential Tfh profile participates in the increased risk of lymphoproliferative disease in pSS compared to associated SS, or with other outcomes, or might be a treatment target, is yet to be determined in future studies.

References

1. FASANI S, MAURO D, MACALUSO F *et al*.: Pathogenesis of primary Sjögren's syndrome beyond B lymphocytes. *Clin Exp Rheumatol* 2020; 38: (Suppl. 126): S315-23.
2. BOMBARDIERI M, ARGYROPOULOU O, FERRO F *et al*.: One year in review 2020: pathogenesis of primary Sjögren's syndrome. *Clin Exp Rheumatol* 2020; 38: (Suppl. 126): S3-9.
3. ZHU Y, ZOU L, LIUR Y: T follicular helper cells, T follicular regulatory cells and autoimmunity. *Int Immunol* 2015; 28: 173-9.
4. KURATA I, MATSUMOTO I, SUMIDA T: T follicular helper cell subsets: a potential key player in autoimmunity. *Immunol Med* 2021; 44: 1-9.
5. BLANCO P, HIDEKI UENO H, SCHMITT N: T follicular helper (Tfh) cells in lupus: Activation and involvement in SLE pathogenesis. *Eur J Immunol* 2016; 46: 281-90.
6. NICOLAS P, RUIZ A, COBO-CALVO A *et al*.: The balance in T follicular helper cell subsets Is Altered in neuromyelitis optica spectrum disorder patients and restored by rituximab. *Front Immunol* 2019; 10: 2686.
7. 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* 2016; 183: 76-89.
8. SAITO M, OTSUKA K, USHIO A *et al*.: Unique phenotypes and function of follicular helper T cells and regulatory T cells in Sjögren's syndrome. *Curr Rheumatol* 2018; 14: 239-245.
9. CHEN W, YANG F, XU G, MA J, LIN J: Follicular helper T cells and follicular regulatory T cells in the immunopathology of primary Sjögren's syndrome. *J Leukoc Biol* 2020; 109: 437-47.
10. LI XY, WU ZB, DING J *et al*.: Role of the frequency of blood $CD4^{(+)}$ $CXCR5^{(+)}$ $CCR6^{(+)}$ T cells in autoimmunity in patients with Sjögren's syndrome. *Biochem Biophys Res Commun* 2012; 422: 238-44.
11. DUPRE A, PASCAUD J, RIVIERE E *et al*.: Association between T follicular helper cells and T peripheral helper cells with B-cell biomarkers and disease activity in primary Sjögren's syndrome. *RMD Open* 2021; 7: e001442.
12. VERSTAPPEN G, NAKSHBANDI U, MOSSEL E *et al*.: Is the T follicular regulatory follicular helper T cell ratio in blood a biomarker for ectopic lymphoid structure formation in Sjögren's syndrome? Comment on the article by Fonseca *et al*. *Arthritis Rheumatol* 2018; 70: 1354-55.
13. SZABO K, PAPP G, DEZSO B, ZEHER M: The histopathology of labial salivary glands in primary Sjögren's syndrome: focusing on follicular helper T cells in the inflammatory infiltrates. *Mediators Inflamm* 2014; 2014: 631787.
14. VERSTAPPEN G, KROESE F, MEINERS P *et al*.: B cell depletion therapy normalizes follicular T cells in primary Sjögren's syndrome. *J Rheumatol* 2017; 44: 49-58.
15. PONTARINI E, MURRAY-BROWN W, CROIA C *et al*.: Unique expansion of IL-21+ Tfh and Tph cells under control of ICOS identifies Sjögren's syndrome with ectopic germinal centres and MALT lymphoma. *Ann Rheum Dis* 2020; 79: 1588-99.
16. FONSECA V, ROMAO V, AGUA-DOCE A *et al*.: The ratio of blood T follicular regulatory cells to T follicular helper cells marks ectopic lymphoid structure formation while activated follicular helper T cells indicate disease activity in primary Sjögren's syndrome. *Arthritis Rheumatol* 2018; 70:7 74-84.
17. ZHANG X, LINDWALL E, GAUTHIER C *et al*.: Circulating CXCR5 þ CD4 þ helper T cells in systemic lupus erythematosus patients share phenotypic properties with germinal center follicular helper T cells and promote antibody production. *Lupus* 2015; 24: 909-17.
18. CHOI JY, HO J, PASOTO SG *et al*.: Circulating follicular helper-like T cells in systemic lupus erythematosus: association with disease activity. *Arthritis Rheumatol* 2015; 67: 988-99.
19. LIN J, YU Y, MA J *et al*.: PD-1+CXCR5-$CD4^+$T cells are correlated with the severity of systemic lupus erythematosus. *Rheumatology* 2019; 58: 2188-92.
20. LIARSKI V, KAVERINA, N, CHANG A *et al*.: Cell distance mapping identifies functional T follicular helper cells in inflamed human renal tissue. *Sci Transl Med* 2014; 6: 230ra246

21. SHIBOSKI CH, SHIBOSKI SC, SEROR R *et al*.: 2016 American College of Rheumatology/ European League Against Rheumatism Classification Criteria for Primary Sjögren's Syndrome: A consensus and data-driven methodology involving three international patient cohorts. *Arthritis Rheumatol* 2017; 69: 35-45.
22. GLADMAN D, UROWITZ M, GOLDSMITH C *et al*.: The reliability of the systemic lupus international collaborating clinics-American College of Rheumatology damage index in patients with systemic lupus erythematosus. *Arthritis Rheum* 1997; 40: 809-13.
23. FURUZAWA-CARBALLEDA J, SÁNCHEZ-GUERRERO J, BETANZOS JL *et al*.: Differential cytokine expression and regulatory cells in patients with primary and secondary Sjögren's syndrome. *Scand J Immunol* 2014; 80: 432-40.
24. SÁNCHEZ-GUERRERO J, AGUIRRE E, PÉREZ M *et al*.: The wafer test: a semi-quantitative test to screen for xerostomia. *Rheumatology* 2002; 41: 381-9.
25. YAO Q, ALTMAN RD, WANG X: Systemic lupus erythematosus with Sjögren syndrome compared to systemic lupus erythematosus alone: a meta-analysis. *J Clin Rheumatol* 2012; 18: 28-32.
26. KOLLERT T, FISHER B: Equal rights in autoimmunity: is Sjögren's syndrome ever "secondary"? *Rheumatology* 2020; 59: 1218-25.
27. PASOTO S, OIVEIRA V, BONFA E: Sjögren's syndrome and systemic lupus erythematosus: links and risks. *Open Access Rheumatol* 2019; 11: 33-45.
28. MANOUSSAKIS MN, GEORGOPOULOU C, ZINTZARAS E *et al*.: Sjogren's syndrome associated with systemic lupus erythematosus: clinical and laboratory profiles and comparison with primary Sjogren's syndrome. *Arthritis Rheum* 2004; 50: 882-91.
29. BOLOAGNA S, NICO N, FLORENZI G, CAVALCNATE W, LORENCO S: Peculiar histopathological features in minor salivary gland in lupus erythematosus. *Lupus* 2018; 27: 1706-11.
30. FERNANDES JD, NICO MM, AOKI V *et al*.: Xerostomia in Sjögren's syndrome and lupus erythematosus: A comparative histological and immunofluorescence study of minor salivary glands alterations. *J Cutan Pathol* 2010; 37: 432-8.
31. HERNÁNDEZ-MOLINA G, AVILA-CASADO C, CÁRDENAS-VELÁZQUEZ F *et al*.: Similarities and differences between primary and secondary Sjögren's syndrome. *J Rheumatol* 2010; 37: 800-8.
32. FURUZAWA J, SANCHEZ-GUERRERO J, BETANZOS E, AVILA C, LLORENTE L, HERNÁNDEZ- MOLINA G: Differential cytokine expression and regulatory cells in patients with primary and secondary Sjögren's syndrome. *Scand J Immunol* 2014; 80: 432-40.
33. UENO H, BANCHEREAU J, VINUESA C: Pathophysiology of T follicular helper cells in humans and mice. *Nat Immunol* 2015; 16: 142-52.
34. KIM JW, LEE J, HONG SM, LEE J, CHO M, PARK S: Circulating $CCR7^{lo}PD\text{-}1^{hi}$ follicular helper T cells indicate disease activity and glandular inflammation in patients with primary Sjögren's syndrome. *Immune Netw* 2019; 19: e26.
35. BARCELOS F, MARTINS C, MADEIRA N *et al*.: Lymphocyte subpopulation in Sjögren's syndrome is distinct in anti-SSA positive patients and related to disease activity. *Clin Rheumatol* 2021; 40: 2791-804.
36. LE COZ C, JOUBLIN A, PASQUALI JL, KORGANOW-AS, DUMORTIER H, MONNEAUX F: Circulating TFH subset distribution is strongly affected in lupus patients with an active disease. *PLoS One* 2013; 8: e75319
37. JIN L, YU D, LI X *et al*.: $CD4^{+}CXCR5^{+}$ follicular helper T cells in salivary gland promote B cells maturation in patients with primary Sjögren's syndrome. *Int J Clin Exp Pathol* 2014; 7: 1988-96.
38. MAEHARA T, MORIYAMA M, HAYASHIDA J *et al*.: Selective localization of T helper subsets in labial salivary glands from primary Sjögren's syndrome patients. *Clin Exp Immunol* 2012; 169: 89-99.
39. AQRAWI L, ICANCHENKO M, BJÖRK A *et al*.: Diminished CXCR5 expression in peripheral blood of patients with Sjögren's syndrome may relate to both genotype and salivary gland homing. *Clin Exp Immunol* 2018; 192: 259-70.
40. XU B, WANG S, ZHOU M *et al*.: The ratio of circulating follicular T helper cell to follicular T regulatory cell is correlated with disease activity in systemic lupus erythematosus. *Clin Immunol* 2017; 183: 46-53.
41. KAPSOGEORGOU E, CHRISTODOULOU M, PANAGIOTAKOS D *et al*.: Minor salivary gland inflammatory lesions in Sjögren's syndrome: do they evolve? *J Rheumatol* 2013; 40: 1566-71.