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**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**

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**Abbreviations:** AID: activation-induced cytidine deaminase; Bcl-6: B cell lymphoma 6 protein; Breg: regulatory B cell; B10: IL-10 producing B cell; B10<sub>PRO</sub>: B10 progenitor cells; CD: Cluster of Differentiation; CCR7: C-C chemokine receptor type 7; CXCL10: C-X-C chemokine ligand 10; C-X-C chemokine receptor 5: CXCR5; DCs: dendritic cells; DN: double-negative; EGMs: extraglandular manifestations; FITC: fluorescein isothiocyanate; GC: germinal centre; ICOS: inducible T cell co-stimulator; IL: interleukin; LSG: labial salivary gland; PD-1: programmed cell death protein 1; PE: R-phycoerythrin; PE-Cy5: R-phycoerythrin-Cyanine dye 5; PerCP-Cy5.5: Peridinin-chlorophyll protein-Cyanine dye 5.5; pSS: primary Sjögren's syndrome; RF: rheumatoid factor; Tr1: type 1 regulatory T cell; SLE: systemic lupus erythematosus; T<sub>FH</sub>: T follicular helper

## Summary

Follicular T helper ( $T_{FH}$ ) 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 primary Sjögren's syndrome (pSS) and systemic lupus erythematosus (SLE), the aim of this study was to profile the pathological connection between peripheral  $T_{FH}$  cells and B cells in the two diseases. Twenty-five pSS patients, 25 SLE patients and 21 healthy controls were enrolled in the study. We determined the ratio of circulating  $T_{FH}$ -like cells, their IL-21 production, and different B cell subsets by flow cytometry. 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 double-negative 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. Correlation analysis between  $T_{FH}$ -like cells and certain B cell subsets revealed possible defects during B cell selection. In conclusion, 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.

## Introduction

Autoimmune diseases are characterized by the breakdown of immune tolerance leading to autoreactive immune mechanisms and consequential tissue and organ damages. In the pathogenesis of autoimmune diseases, various cellular and humoral immune processes have been described, including disproportional T and B cell responses, altered cytokine milieu and disturbed apoptotic processes, which result in exaggerated immune responses. In the last decades, a large amount of studies confirmed that B cell activation plays a crucial role in the inflammatory processes through antigen presentation, autoantibody production and secretion of numerous pro-inflammatory factors. However, besides the established disease-promoting role of B cells, certain B cell subsets, so-called regulatory B (Breg) cells have a negative regulatory effect by producing regulatory cytokines such as IL-10, and directly interacting with activated T cells via cell-to-cell contact <sup>1</sup>.

The proliferation and differentiation of B cells highly depend on their collaboration with a special subset of CD4<sup>+</sup> T cells in the germinal centres (GCs) of secondary lymphoid organs. These recently described T cells, so-called follicular helper T (T<sub>FH</sub>) cells, are generated from peripheral naive CD4<sup>+</sup> T cells in the T cell zone of lymphoid organs. The proper interplay of activated B cells and T<sub>FH</sub> cells is essential for the development of extrafollicular short-lived, low-affinity plasma cells and also for GC responses. Within GCs, T<sub>FH</sub> cells facilitate the generation of high-affinity memory B cells and long-lived plasma cells. IL-21 is the hallmark cytokine of T<sub>FH</sub> cells and has been shown to function as a potent inducer of plasma cell formation; moreover, it is involved in GC B cell selection, as well <sup>2</sup>.

Considering the critical role of  $T_{FH}$  cells and their IL-21 cytokine secretion in B cell activation and antibody production, their failure to maintain self-tolerance and potential contribution to autoimmunity drew an intensive attention. Recent investigations shed light on altered  $T_{FH}$  profiles in various autoimmune conditions. In systemic lupus erythematosus (SLE), which is a prototypic systemic autoimmune disease characterised by immune complex-mediated systemic tissue damage, it is well established that the imbalance of different subsets of B cells is crucial for the initiation and perpetuation of the disease. Recently, elevated percentages of peripheral  $CD4^+ICOS^{high}$  and  $CD4^+CXCR5^+ICOS^{high}$   $T_{FH}$  cells were reported in SLE patients. Notably, these cell proportions showed associations with autoantibody titres and the presence of glomerulonephritis, as well <sup>3</sup>. Moreover, corticosteroid pulse therapy down-regulated the number of circulating  $T_{FH}$  cells, indicating that  $T_{FH}$  cells may be good therapeutic targets in the disease <sup>4</sup>.

In primary Sjögren's syndrome (pSS), which is a common systemic autoimmune disease characterized by chronic inflammation and consequential destruction of exocrine glands, humoral autoimmune responses, B cell activation and autoantibody production are key immune abnormalities, as well. Immunohistological analysis of biopsies from minor salivary glands commonly demonstrates the presence of ectopic GCs in pSS. The number of GCs in salivary glands correlates with the severity of inflammation, and anti-SSA/Ro and anti-SSB/La autoantibody-production. Moreover, the formation of ectopic GCs carries a higher risk of developing B cell lymphoma <sup>5</sup>. Recently, we demonstrated elevated circulating  $CD4^+CXCR5^+ICOS^+PD-1^+$   $T_{FH}$  cell percentages in pSS. Based on our data, we found a strong association between the  $T_{FH}$  cell proportions and the presence of systemic extraglandular manifestations (EGMs) and

anti-SSA/SSB autoantibody positivity. Patients with higher T<sub>FH</sub> cell proportions also had elevated serum levels of IL-12 and IL-21 <sup>6</sup>. Maehara et al. described that the expression of Th2 and certain T<sub>FH</sub>-related molecules was associated with robust lymphocytic accumulation and ectopic GC formation <sup>7</sup>. We also investigated the localization of T<sub>FH</sub> cells in the lymphocyte infiltration in labial salivary gland (LSG) biopsies gained from pSS patients at the time of disease onset, and found that T<sub>FH</sub> cell markers (CD84, PD-1, and Bcl-6) occurred predominantly in more organized lymphoid structures with higher focus scores <sup>8</sup>, which indicates that T<sub>FH</sub> cells may be good therapeutic targets in pSS, as well.

However, the complex interplay between T<sub>FH</sub> cells and the special subsets of B cells is not yet fully elucidated. Since identification of the pathogenic pathways and the corresponding biomarkers linking abnormal cellular activity to disease activity and outcome is essential for defining proper therapeutic targets, the aim of the present study was to profile the pathological connection between the peripheral T<sub>FH</sub> cells and B cell subsets and the clinical features of pSS and SLE.

## Materials and methods

### *Patients and healthy individuals*

Total of 25 patients with pSS (24 female and 1 male; mean age:  $57.88 \pm 9.19$  years) and 25 patients with SLE (24 female and 1 male; mean age:  $41.17 \pm 13.20$  years) were enrolled in the study. All patients were recruited from the Outpatient Clinic for systemic autoimmune diseases at the Division of Clinical Immunology, University of Debrecen, where they received regular follow-up treatment. The average disease duration in case of pSS was  $13.40 \pm 7.94$  years, while  $11.44 \pm 9.14$  years in SLE. The diagnosis of pSS was based on the European-American consensus criteria <sup>9</sup>. Among pSS patients, 15 suffered from extraglandular manifestations (EGMs), while 10 had only glandular symptoms. The distribution of EGMs of pSS patients were as follows: polyarthralgia n=11, Raynaud's phenomenon n=7, polyarthritis n=6, vasculitis n=2. The exclusion criteria included therapy with immunosuppressive/immunomodulant agents. Vasculitis or other EGMs needing immunosuppressive treatment were newly recognised. All patients with SLE fulfilled the corresponding diagnostic criteria for lupus <sup>10, 11</sup>; 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≥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 dose of the treatment did not exceed 8 mg methylprednisolone per day in any case of SLE patients. The blood samples were collected from SLE patients 24 hours after taking the regular methylprednisolone medication. The active disease status in the corresponding SLE patients was newly recognised, and these patients undergone the blood sampling before

any changes in their therapy. In addition, patients were also categorized according to their serological markers with a special emphasis on the presence of autoantibodies. Among pSS patients, we found 9 anti-Ro/SSA positive (SSA>10 U/ml) individuals, 4 of them were anti-Ro/SSA – anti-La/SSB double positive. Regarding SLE patients, 15 of them were anti-dsDNA positive (dsDNA>20 IU/ml). The control group consisted of 21 age- and sex-matched (20 female and 1 male; mean age: 39.10 ± 12.43years) healthy volunteers. No patients or controls enrolled in this study had ongoing infections, either viral or bacterial. Informed written consent was obtained from all subjects, and the study was approved by the Ethics Committee of the University of Debrecen. All experiments carried out were in compliance with the Declaration of Helsinki.

#### *Cell surface staining and flow cytometric analysis*

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinised venous blood sample by Ficoll-Histopaque (Sigma-Aldrich, St Louis, MO, USA) density-gradient centrifugation. Cells were then harvested and washed twice and stained for 20 min at 4°C using specific antibodies. For identification of naive and memory B cell subsets, we used IgD-fluorescein isothiocyanate (FITC) (clone: IADB6)/CD27-phycoerythrin (PE) (clone: 1A4CD27)/CD19-phycoerythrin-Cyanine dye 5 (PE-Cy5) (clone: J3-119) (all from Beckman Coulter Inc, Fullerton, CA, USA and Immunotech, Marseille, France). To identify naive, mature-naive, primarily memory and transitional B cell subpopulations, cells were stained with the following combination of monoclonal antibodies: CD38-FITC (clone: HIT2)/CD27-PE/CD19-PE-Cy5/CD24-allophycocyanin (APC) (ML5) (BD Biosciences, San Diego, CA, USA and Beckmann Coulter and BioLegend, San Diego, CA, USA). At least 20.000 CD19<sup>+</sup> events of each sample were analysed within the whole lymphocyte population. For the assessment of circulating T<sub>FH</sub>



cells we used CXCR5-Alexa Fluor 488 (clone: RF8B2)/ICOS-PE (clone: DX29)/PD-1-Peridinin-chlorophyll protein-Cyanine dye 5.5 (PerCP-Cy5.5) (clone: EH12.1)/CD4-APC (clone: RPA-T4) (all from BD Biosciences) monoclonal antibodies. For the determination of naive and activated or memory CD4<sup>+</sup> T cell subsets we used CD45RA-PE (clone: HI100) and CD45RA-PerCP-Cy5.5 (clone: HI100) monoclonal antibodies (both from BioLegend). Fluorescence Minus One controls were used in all procedures. The stained cells were measured by FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and data were analysed using FlowJo Software (Treestar, Ashland, OR, USA). In case of T<sub>FH</sub> cells at least 75.000 CD4<sup>+</sup> events per sample were analysed within the whole lymphocyte population.

#### *Intracellular cytokine analysis by flow cytometry*

The cytoplasmic IL-21 content of circulating T<sub>FH</sub> cells was also determined by flow cytometry. Briefly, isolated PBMCs were cultured in modified RPMI 1640 medium with GLUTAMAX<sup>TM</sup>-I (Life Technologies Corporation, Carlsbad, CA, USA) supplemented with 100 U/ml penicillin, 100 ng/ml streptomycin and 10% heat-inactivated fetal calf serum (Life Technologies) at a concentration of 2x10<sup>6</sup>/ml in 24-well tissue culture plates for analysing single-cell cytokine production. Total PBMCs were incubated with phorbol-12-myristate 13-acetate (PMA) (25 ng/ml), ionomycin (1 µg/ml), and Golgi Stop brefeldin-A (10 µg/ml) (all from Sigma Aldrich) for 5 h at 37°C in 5% CO<sub>2</sub> milieu. Cell surface staining was performed with a cocktail of CXCR5-AF488, PD-1-PerCP-Cy5.5 and CD4-APC monoclonal antibodies for 20 min at 4°C. The cells then fixed and permeabilized with Intraprep<sup>TM</sup> permeabilization reagent (Beckman Coulter Inc, Miami, FL, USA) according to the manufacturer's instructions,

and intracellular cytokines were stained with anti-IL-21-PE (clone: 3A3-N2.1) (BD Biosciences).

For intracellular IL-10 induction of B cells, PBMCs were stimulated with CpG (Toll-like receptor 9 ligand, ODN 2006 type B; 0.5  $\mu$ M/ml; Hycult Biotech Inc., Uden The Netherlands) for 48 h at 37°C in 5% CO<sub>2</sub> milieu. For the last 5 h, PMA (25 ng/ml), ionomycin (1  $\mu$ g/ml), and Golgi Stop brefeldin-A (10  $\mu$ g/ml) were added to the culture. Cells were then harvested, washed in staining buffer, and incubated for 20 min at 4°C with CD19-PE-Cy5. Intracellular staining method was performed with IL-10-PE (clone: JES3-9D7) as described above. Measurements were performed and data were collected by FACS Calibur flow cytometer and data were analysed using FlowJo Software. At least 75.000 CD4<sup>+</sup> events or 15.000 CD19<sup>+</sup> events of each sample were analysed within the entire lymphocyte population. The viability of the CpG exposed cells were determined by 7-aminoactinomycin-D (7-AAD; BioLegend) staining with flow cytometric analysis. The identification of B cells was performed with CD19-FITC (clone J3-119) monoclonal antibody. The percentages of dead cells among the CD19<sup>+</sup> B cells were increased in the PIB and CpG+PIB group, however the average percentages of dead cells remained <2.5 %. Regarding the whole lymphocyte group, the proportions of dead cells did not exceed 5% on average (Fig S1).

#### *Assessment of anti-dsDNA, anti-Ro/SSA and anti-La/SSB autoantibodies*

As part of the routine diagnostic evaluation, autoantibodies were determined by ELISA technique with AUTOSTAT II kits (Hycor Biomedical, Indianapolis, IN, USA) according to the manufacturer's instructions.

*Statistical analysis*

Data were represented and statistical analysed with GraphPad Prism 5 software (Graphpad Software, San Diego, USA). Data are presented as mean  $\pm$  SD. 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  $p < 0.05$ .

## Results

### *The distribution of peripheral B cell subpopulations*

According to the expression of IgD, CD27, CD38 and CD24 cell surface markers, the following B cell subsets were identified: CD19<sup>+</sup>IgD<sup>+</sup>CD27<sup>-</sup> naive B cells, CD19<sup>+</sup>IgD<sup>+</sup>CD27<sup>+</sup> non-switched memory B cells, CD19<sup>+</sup>IgD<sup>-</sup>CD27<sup>+</sup> switched memory B cells, CD19<sup>+</sup>IgD<sup>-</sup>CD27<sup>-</sup> double negative (DN) B cells, CD19<sup>+</sup>CD38<sup>-</sup>CD24<sup>hi</sup>CD27<sup>+</sup> primarily memory B cells, CD19<sup>+</sup>CD38<sup>hi</sup>CD24<sup>hi</sup>CD27<sup>-</sup> transitional B cells, CD19<sup>+</sup>CD38<sup>+</sup>CD24<sup>+</sup> mature-naive B cells and CD19<sup>+</sup>CD38<sup>hi</sup>CD27<sup>hi</sup> plasmablasts. Cells were quantified as their percentage in the CD19<sup>+</sup> lymphocyte population.

Regarding CD19<sup>+</sup>IgD<sup>-</sup>CD27<sup>+</sup> 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,  $p=0.0005$ ) (Fig. 1a). Correspondingly, both subgroups of pSS patients had significantly lower percentages compared to controls (pSS without EGMs vs. control:  $17.30 \pm 5.06\%$  vs.  $23.23 \pm 6.78\%$ , respectively,  $p=0.0207$ ; pSS with EGMs vs. control:  $13.58 \pm 8.83\%$  vs.  $23.23 \pm 6.78\%$ , respectively,  $p=0.0007$ ) (Fig. 1a). On the contrary, CD19<sup>+</sup>IgD<sup>-</sup>CD27<sup>+</sup> 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<sup>+</sup>IgD<sup>-</sup>CD27<sup>+</sup> 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<sup>+</sup>IgD<sup>+</sup>CD27<sup>+</sup> 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,  $p=0.0019$ ) (Fig. 1b). This observation was also valid for SLEDAI $<6$  and SLEDAI $>6$

patient subgroups separately, as well ( $13.21 \pm 11.88\%$  vs.  $21.64 \pm 11.52\%$ , respectively,  $p=0.0333$  and  $6.25 \pm 5.03\%$  vs.  $21.64 \pm 11.52\%$ , respectively,  $p<0.0001$ ) (Fig. 1b).

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,  $p=0.0290$ ). However, intragroup differences could be found only in pSS patients with EGMs when compared to controls ( $3.306 \pm 3.030\%$  vs.  $3.796 \pm 1.681\%$ , respectively,  $p=0.0291$ ) (Fig. 1c). Interestingly, in contrast to measurements in pSS, the percentages of  $CD19^+IgD^-CD27^-$  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,  $p=0.0119$ ). Nevertheless, this significant change could be detected only in SLEDAI<6 patient subgroup compared to controls ( $7.017 \pm 4.998\%$  vs.  $3.796 \pm 1.681\%$ , respectively,  $p=0.0204$ ) (Fig. 1c).

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,  $p=0.0261$ ); however, this significant elevation could be observed only in pSS patients without EGMs ( $63.70 \pm 14.14\%$  vs.  $51.32 \pm 15.14\%$ , respectively,  $p=0.0382$ ) (Fig. 1d). 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,  $p=0.0471$ ) (Fig. 1d). Certainly, this tendency was also found in the remaining patient subgroups, however the differences were not significant.

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,  $p=0.0289$ ) (Fig. 1e), of note, this difference was observed between pSS with EGMs and healthy subjects. The frequency of  $CD19^+CD38^-$

CD24<sup>hi</sup>CD27<sup>+</sup> 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,  $p=0.0042$ ) (Fig. 1e).

There was no difference in the pSS groups regarding the distribution of both CD19<sup>+</sup>CD38<sup>hi</sup>CD24<sup>hi</sup>CD27<sup>-</sup> and CD19<sup>+</sup>CD38<sup>+</sup>CD24<sup>+</sup> B cell subsets. However, this could be due to the low number of patients in the sub-divided groups. On the other hand, we found that the ratio of CD19<sup>+</sup>CD38<sup>hi</sup>CD24<sup>hi</sup>CD27<sup>-</sup> 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,  $p=0.0499$ ) (data not shown). In the overall SLE group and in patients with SLEDAI>6, the frequency of CD19<sup>+</sup>CD38<sup>hi</sup>CD24<sup>hi</sup>CD27<sup>-</sup> 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,  $p=0.0323$ ) (Fig. 1f). Furthermore, the ratio of CD19<sup>+</sup>CD38<sup>+</sup>CD24<sup>+</sup> 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,  $p=0.0094$ ) (Fig. 1f).

The ratio of peripheral CD19<sup>+</sup>CD38<sup>hi</sup>CD27<sup>hi</sup> 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,  $p=0.0014$ ) (Fig. 1h). However, in patients with SLE, we found significantly higher frequency of CD19<sup>+</sup>CD38<sup>hi</sup>CD27<sup>hi</sup> plasmablasts only in

SLEDAI>6 group compared to healthy subjects ( $1.150 \pm 1.181\%$  vs.  $0.2671 \pm 0.2932\%$ , respectively,  $p=0.0403$ ) (Fig. 1h).

#### *Quantification of circulating T<sub>FH</sub>-like cells*

Circulating T<sub>FH</sub>-like cells were quantified as their percentages within the CD4<sup>+</sup> lymphocytes of peripheral blood. Within CD4<sup>+</sup>CXCR5<sup>+</sup> lymphocytes, we also determined the fraction of ICOS<sup>+</sup>PD-1<sup>+</sup> T cells. According to our results, the percentages of CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>+</sup>PD-1<sup>+</sup> T<sub>FH</sub>-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,  $p=0.0218$ ) (Fig. 2a). The frequency of T<sub>FH</sub>-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,  $p=0.0184$ ) (Fig. 2a). Additionally, we analysed the distribution of T<sub>FH</sub> cells percentages in the patient groups according to the absence or presence of specific autoantibodies. The ratio of T<sub>FH</sub> cells showed a significant 2-fold increase in anti-Ro/SSA antibody-positive group compared to healthy controls and anti-Ro/SSA antibody-negative group as well ( $0.4806 \pm 0.2699\%$  vs.  $0.2235 \pm 0.0979\%$ , respectively,  $p=0.0050$  and  $0.4806 \pm 0.2699\%$  vs.  $0.2568 \pm 0.1893\%$ , respectively,  $p=0.0138$ ) (Fig. 2b). 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,  $p=0.0192$ ) (Fig. 2b).

We also determined CD45RA<sup>+</sup> naive CD4<sup>+</sup> T cells and CD45RA<sup>-</sup> activated or memory CD4<sup>+</sup> T cells in a smaller groups of patients and controls (pSS n=9, SLE n=8 and

control n=6). Then we measured the ratio of CXCR5<sup>+</sup>ICOS<sup>+</sup> or CXCR5<sup>+</sup>PD-1<sup>+</sup> cells in both CD4<sup>+</sup>CD45RA<sup>-</sup> and CD4<sup>+</sup>CD45RA<sup>+</sup> subsets. Naive CD45RA<sup>+</sup> T cells mostly showed reduced ICOS and PD-1 expression compared to activated or memory CD45RA<sup>-</sup> T cells. The ratio of CXCR5<sup>+</sup>ICOS<sup>+</sup> cells were increased in patients with pSS and SLE in the CD4<sup>+</sup>CD45RA<sup>+</sup> subset, although the values were generally under 0.25%. The percentages of CXCR5<sup>+</sup>ICOS<sup>+</sup> cells were elevated especially in SLE and the values varied between 0.120-1.310%. The ratios of CXCR5<sup>+</sup>PD-1<sup>+</sup> cells were also increased in both disease and showed similar tendency: the values were generally under 0.68% in the CD4<sup>+</sup>CD45RA<sup>+</sup> subset, while those were between 1.280-12.10% in the CD4<sup>+</sup>CD45RA<sup>-</sup> T cells. Moreover, we investigated the association between the ratios of CXCR5<sup>+</sup>ICOS<sup>+</sup> cells and CXCR5<sup>+</sup>PD-1<sup>+</sup> cells in the CD4<sup>+</sup>CD45RA<sup>-</sup> subsets, as well, and we found a significant positive correlation in the whole measured group (R=0.6393, respectively, p=0.0010) (Fig. S2).

#### *Measurement of individual cytokine production of circulating T<sub>FH</sub> cells*

To establish the cytokine profile of circulating T<sub>FH</sub>-like cells, we determined intracellular IL-21 cytokine production. The frequency of IL-21 producing CD4<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>+</sup> cells did not differ significantly between the whole group of pSS patients and healthy controls. However, we found that the percentages of CD4<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>+</sup>IL-21<sup>+</sup> T cells were significantly higher in pSS patients with EGMs than control values (0.2449 ± 0.1657% vs. 0.1469 ± 0.0649%, respectively, p=0.0442) (Fig. 2c). The ratio of IL-21 producing T<sub>FH</sub>-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 ± 0.1886% vs. 0.1469 ± 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,  $p=0.0123$ ) (Fig. 2c). 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,  $p=0.0297$ ) (Fig. 2d). 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,  $p=0.0489$ ) (Fig. 2d). Additionally, we investigated the possible associations between the ratio of  $CD4^+CXCR5^+ICOS^+PD-1^+$   $T_{FH}$ -like cells and IL-21 producing  $CD4^+CXCR5^+PD-1^+$  T cells. A significant positive correlation was found in pSS patient group ( $R=0.5915$ , respectively,  $p=0.0018$ ) and a positive, but not significant association observed in SLE patient group ( $R=0.3862$ , respectively,  $p=0.0566$ ) (Fig. 3).

#### *Assessment of IL-10 producing B cells*

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. After 5-hour incubation with PIB + CpG, we found no significant differences in the ratio of  $IL-10^+CD19^+$  B cells between patient groups and healthy controls (Fig. 4a). However, after 48 h incubation period and stimulation with PIB alone for the last 5 h, we found that the percentages of  $IL-10^+CD19^+$  B cells in the pSS patient group were

significantly decreased than those measured in controls and SLE patient group ( $0.9688 \pm 0.4284\%$  vs.  $1.592 \pm 0.8799\%$ , respectively,  $p=0.0076$  and  $0.9688 \pm 0.4284\%$  vs.  $1.645 \pm 1.198\%$ , respectively,  $p=0.0127$ ) (Fig. 4b). Furthermore, IL-10<sup>+</sup>CD19<sup>+</sup> B cell percentages were determined after in vitro B10<sub>PRO</sub> cell maturation by stimulation with CpG for 48 h with PIB added for the final 5 h to the culture. The total frequency of IL-10 producing CD19<sup>+</sup> B cells including B10 and matured B10<sub>PRO</sub> cells, was significantly elevated compared with PIB alone treated cells in case of each equivalent groups (control:  $5.543 \pm 2.372\%$  vs.  $1.592 \pm 0.8799\%$ , respectively,  $p<0.0001$ ; pSS:  $5.712 \pm 0.3310\%$  vs.  $0.9688 \pm 0.4284\%$ , respectively,  $p<0.0001$  and SLE:  $6.342 \pm 4.023\%$  vs.  $1.645 \pm 1.198\%$ , respectively,  $p<0.0001$ ) (Fig. 4b). However, there were no significant differences between the patients groups and healthy controls regarding the distribution of IL-10<sup>+</sup>CD19<sup>+</sup> B cells after 48 h of CpG stimulation.

*Correlation analysis between peripheral T<sub>FH</sub>-like cells and certain B cell subpopulations in pSS*

We measured the relationship between the percentages of CD19<sup>+</sup>CD38<sup>hi</sup>CD24<sup>hi</sup>CD27<sup>+</sup> transitional B cells and the proportions of both CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>+</sup>PD-1<sup>+</sup> T<sub>FH</sub>-like cells and CD4<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>+</sup>IL-21<sup>+</sup> T<sub>FH</sub> cells in pSS. We revealed a positive but not significant correlation between the ratio of transitional and T<sub>FH</sub>-like cells ( $R=0.3389$ , respectively,  $p=0.0975$ ), moreover a significant positive correlation between transitional B cells and IL-21 producing T<sub>FH</sub> cells in the overall pSS group ( $R=0.4455$ , respectively,  $p=0.0256$ ) (data not shown). When we focused only on pSS patients with EGMs, we revealed strong significant positive correlations between the aforementioned cell subsets (transitional B vs. T<sub>FH</sub>-like cells:  $R=0.5964$ , respectively,  $p=0.0189$  and transitional B

vs. IL-21<sup>+</sup> T<sub>FH</sub>-like cells R=0.5857, respectively, p=0.0218) (Fig. 5a and 5b). Similar observations were found between the ratios of CD19<sup>+</sup>CD38<sup>+</sup>CD24<sup>+</sup> mature-naive B and CD4<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>+</sup>IL-21<sup>+</sup> T<sub>FH</sub> cells in pSS. A significant positive correlation was found in the whole pSS group (R=0.5058, respectively, p=0.0095; data not shown) and in patients with EGMs as well, nevertheless the association was more powerful in the latter case (R=0.5536, respectively, p=0.0323) (Fig. 5c).

#### *Correlation analysis between peripheral T<sub>FH</sub>-like cells and certain B cell subsets in SLE*

We measured the association between CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>+</sup>PD-1<sup>+</sup> T<sub>FH</sub>-like cells and both CD19<sup>+</sup>IgD<sup>-</sup>CD27<sup>-</sup> DN B cells and CD19<sup>+</sup>CD38<sup>-</sup>CD24<sup>hi</sup>CD27<sup>+</sup> primarily memory B cells in SLE. A significant negative correlation was observed between the percentages of DN B cells and T<sub>FH</sub>-like cells in SLE patients with SLEDAI<6 (R=-0.5758, respectively, p=0.0156) (Fig. 5d). Furthermore, a significant negative correlation was found between the ratios of primarily memory B cells and T<sub>FH</sub>-like cells also in the same subgroup (R=-0.6317, respectively, p=0.0065) (Fig. 5e). Of note, these differences were tendentially observed in the whole SLE group as well, however the correlations were not significant (data not shown). On the other hand, a significant positive correlation revealed between the proportions of CD4<sup>+</sup>CXCR5<sup>+</sup>IL-21<sup>+</sup> T cells and CD19<sup>+</sup>CD38<sup>hi</sup>CD27<sup>hi</sup> plasmablasts in the whole SLE patient group (R=0.4110, respectively, p=0.0412) (Fig. 5f).

#### *Association of peripheral T<sub>FH</sub>-like cells, B cell subsets with serological parameters*

We examined the possible relationship between circulating T<sub>FH</sub>-like cells or IL-21 producing T<sub>FH</sub>-like cells and serological markers (Table 1), moreover between certain B cell subsets and serological markers as well (Table 2).

Accepted Article

## Discussion

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<sup>+</sup>IgD<sup>+</sup>CD27<sup>-</sup> naive B cells in the peripheral blood of both pSS and SLE patients compared to healthy individuals. This observation is consistent with previous reports<sup>12-14</sup> and 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<sup>15,16</sup>.

There is another major tolerance checkpoint during the maturation stage of immature B cells when transitional B cells overcome a negative selection. In healthy adults, only a little portion of peripheral B cells are CD19<sup>+</sup>CD38<sup>hi</sup>CD24<sup>hi</sup>CD27<sup>-</sup> transitional B cells and most of them belong to the mature-naive and memory B cell pool. The pathologic accumulation of these cells may occur due to their increased exiting from the bone marrow or disturbed entrance into secondary lymphoid organs<sup>17</sup>. In accordance with previous findings<sup>17-19</sup>, 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 correlated 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.

When the transitional B cells undergo maturation processes, mature-naive B cells are generated which circulate into B cell follicles in secondary lymphoid organs<sup>20</sup>. Of note, the defect in early self-tolerance may also cause the expansion of circulating self-reactive and polyreactive type of mature-naive B cell subset. In our study, we measured significantly higher percentages of CD19<sup>+</sup>CD38<sup>+</sup>CD24<sup>+</sup> mature-naive B cells in SLE. Importantly, large numbers of autoreactive B cells occur among the mature-naive B cell compartment in SLE<sup>21</sup>.

We also confirmed that peripheral CD19<sup>+</sup>IgD<sup>+</sup>CD27<sup>+</sup> non-switched memory B cells and CD19<sup>+</sup>IgD<sup>-</sup>CD27<sup>+</sup> switched memory B cells are strongly diminished in both pSS and SLE<sup>17, 22-24</sup>. 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<sup>12, 13, 25</sup>. Recent findings indicate that CD19<sup>+</sup>IgD<sup>+</sup>CD27<sup>+</sup> non-switched memory B cells from SLE patients are in an activated state and exhibit elevated level of activation induced cytidine deaminase (AID), which promotes their differentiation into IgG-secreting plasma cells<sup>12</sup>. The lower ratio of CD19<sup>+</sup>IgD<sup>-</sup>CD27<sup>+</sup> switched memory B cells in pSS can be also explained by the

pronounced differentiation toward plasma cells or by the shed off CD27 from the surface of memory B cells <sup>26</sup>. We also identified CD19<sup>+</sup>CD38<sup>+</sup>CD24<sup>hi</sup>CD27<sup>+</sup> primarily memory B cells which similar to non-switched memory B cells showed a significant elevation in SLE patients with active disease status.

We detected significantly increased CD19<sup>+</sup>IgD<sup>-</sup>CD27<sup>-</sup> 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 <sup>27, 28</sup>. The proportion of CD19<sup>+</sup>CD38<sup>hi</sup>CD27<sup>hi</sup> 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 <sup>29-31</sup>. 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.

T<sub>FH</sub> cells are crucial immune regulators in secondary lymphoid follicles by controlling B cell proliferation and differentiation. Since the measurement of human GC T<sub>FH</sub> cell from lymphoid follicles could be hardly performed, the investigation of circulating T<sub>FH</sub>-like cells are widely accepted. In the last few years, a number of observations indicated

that circulating T<sub>FH</sub> cells represent a partial differentiated state, however they express T<sub>FH</sub>-related markers at lower intensity and maintain functional characteristic to promote B cell differentiation<sup>32, 33</sup>. At the periphery, T<sub>FH</sub>-like cells may represent a memory CD4<sup>+</sup> T cell condition, and according to certain cell surface molecules, including ICOS, PD-1 and CCR7, they can be subdivided into distinct subsets<sup>34</sup>. In the present study, we revealed a significant increase in the proportion of peripheral CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>+</sup>PD-1<sup>+</sup> T<sub>FH</sub>-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. With this independent set of pSS cases, we reinforced our previous findings<sup>6</sup>. When we divided patients into subgroups based on the presence of anti-SSA/Ro, we found significantly higher T<sub>FH</sub>-like cell percentages in autoantibody positive group. The same changes were measured in SLE, and circulating T<sub>FH</sub>-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.

As next step, we evaluated the proportion of IL-21 producing CD4<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>+</sup> T<sub>FH</sub>-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. Previous investigations on T<sub>FH</sub>-like cells led to similar results<sup>35-41</sup>, however the identification of these cell types are not fully characterized yet. For that reason, some differences could arise with regard to the number of cell surface markers or the exact percentages of T<sub>FH</sub>-like cells; nevertheless



there is broad agreement in that the expansion of T<sub>FH</sub>-like cells in peripheral blood could reflect the severity of autoimmune disorders.

To get a better view on the significance of T<sub>FH</sub>-like cell expansion in pSS and SLE, we analysed the association between the proportion of T<sub>FH</sub>-like cells and other parameters.

Our results revealed a positive correlation between circulating T<sub>FH</sub>-like cells and serum levels of IgG and RF. Additionally, IL-21 producing T<sub>FH</sub>-like cells showed a positive association with serum levels of circulating immune complexes in both pSS and SLE patients. These parameters are strongly related to the regulation of plasma cell generation which underlines the role of T<sub>FH</sub> cells in autoimmune diseases. Regarding B cell subsets, the frequency of transitional B cells correlated with the increased T<sub>FH</sub>-like cell and IL-21<sup>+</sup> T<sub>FH</sub>-like cell percentages in pSS; furthermore, the level of mature-naive B cells also correlated with the increased ratio of T<sub>FH</sub>-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. The mutual cooperation between B cells and T<sub>FH</sub> cells potentially contributes to the development of characteristic pSS features. On the other hand, the expansion of circulating T<sub>FH</sub>-like cells negatively correlated with both primarily memory B cells and DN B cells in SLE patients, which indicates the role of T<sub>FH</sub> cells in B cell differentiation skewing toward plasma cell direction. By demonstrating a positive association between IL-21 producing CD4<sup>+</sup>CXCR5<sup>+</sup> T cells and plasmablast, we underlined the importance of IL-21 in the pathogenesis of SLE.

In the present study, we analysed the whole population of IL-10 producing B cells. The decreased IL-10 producing Breg cell percentages in pSS may elucidate our previous observations that despite of the increased peripheral IL-10 producing regulatory T (Tr1)

cell proportions, a significant decrease in soluble IL-10 level can be detected in the disease. We assume that along with the decrease IL-10 production of Breg cells, the intensification of a counterbalance-mechanism appears; thus, levels of IL-10 producing Tr1 cells increase as a feedback process attempting to compensate the progression of disproportional immune responses<sup>42</sup>.

In conclusion, our observations underline the essential role of the profound expansion of circulating T<sub>FH</sub>-like cells and their IL-21 production in combination with the characteristic abnormal distribution of peripheral B cell subsets disturbances in the pathogenesis of both pSS and SLE (Figure 5). We believe that these extensive experiments revealed the importance of T<sub>FH</sub> cells in these autoimmune diseases and provide valuable insight to invent novel therapeutic strategies targeting certain B cell subsets and suppressing T<sub>FH</sub> mediated responses.

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KSz and GP participated in the study design, performed laboratory experiments, collected, statistically analysed and interpreted the data, and drafted the manuscript.

ASz and TT participated in the interpretation of clinical data. MZ designed the study, interpreted the data and approved the final version of the manuscript for publication. All authors read and approved the final manuscript. Experimental work was performed by the support of the Hungarian National Scientific Research Fund (OTKA Grant No. K101470) and the TAMOP 4.2.2.A-11/1/KONV-2012-0023 “DEFENSE-NET” project.

### **Conflict of Interest**

The authors declare that they have no competing interests.

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### Figure legends

**Figure 1.** Comparative analysis of peripheral B cell subsets from pSS patients, SLE patients and healthy individuals. PBMCs were isolated from 25 pSS patients, 25 SLE patients and 21 healthy controls then were stained with labelled antibodies as described previously. Peripheral blood B cell subsets were quantified as their percentage in CD19<sup>+</sup> lymphocyte population. Representative IgD-CD27 and CD38-CD24 dot plots indicating the distribution and percentages of different B cell subsets. (a) Percentages of CD19<sup>+</sup>IgD<sup>-</sup>CD27<sup>+</sup> switched memory B cells in the overall pSS patient population (n=25), pSS with only glandular symptoms (n=10), pSS with EGMs (n=15), total SLE patient group (n=25), SLE with SLEDAI<6 (n=17), SLE with SLEDAI>6 (n=8) and control subjects (n=21). (b) Frequency of CD19<sup>+</sup>IgD<sup>+</sup>CD27<sup>+</sup> non-switched memory B cells. (c) Proportions of CD19<sup>+</sup>IgD<sup>-</sup>CD27<sup>-</sup> DN B cells. (d) Ratio of CD19<sup>+</sup>IgD<sup>+</sup>CD27<sup>-</sup> naive B cells. (e) Distribution of CD19<sup>+</sup>CD38<sup>-</sup>CD24<sup>hi</sup>CD27<sup>+</sup> primarily memory B cells. (f) Percentages of CD19<sup>+</sup>CD38<sup>hi</sup>CD24<sup>hi</sup>CD27<sup>-</sup> transitional B cells. (g) Proportion of CD19<sup>+</sup>CD38<sup>+</sup>CD24<sup>+</sup> mature-naive B cells. (h) Frequency of CD19<sup>+</sup>CD38<sup>hi</sup>CD27<sup>hi</sup> plasmablasts. Each data point represents an individual subject, horizontal lines show the mean values with SD. Statistically significant differences are indicated by \*, p < 0.05; \*\*, p < 0.01; \*\*\*, p < 0.001; \*\*\*\*, p < 0.0001.

**Figure 2.** Enumeration of circulating T<sub>FH</sub>-like cells in pSS patients, SLE patients and healthy subjects. PBMCs were isolated from 25 pSS patients, 25 SLE patients and 21 healthy controls then were stained with labelled antibodies as described previously. Circulating T<sub>FH</sub>-like cells were assessed as their percentage in CD4<sup>+</sup> lymphocyte population. Representative dot plots are shown the frequency of ICOS<sup>+</sup>PD-1<sup>+</sup> and PD-

IL-21<sup>+</sup> cells within the CD4<sup>+</sup>CXCR5<sup>+</sup> cell population. (a) Percentages of CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>+</sup>PD-1<sup>+</sup> T<sub>FH</sub>-like cells in the overall pSS patient population (n=25), pSS with only glandular symptoms (n=10), pSS with EGMs (n=15), total SLE patient group (n=25), SLE with SLEDAI<6 (n=17), SLE with SLEDAI>6 (n=8) and control subjects (n=21). (b) Ratio of CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>+</sup>PD-1<sup>+</sup> T<sub>FH</sub>-like cells according to the presence of autoantibodies; in anti-Ro/SSA antibody-negative group (n=16), anti-Ro/SSA antibody-positive group (n=9), anti-dsDNA negative group (n=10), anti-dsDNA positive group (n=15) and control subjects (n=21). (c) Distribution of IL-21 producing CD4<sup>+</sup>CXCR5<sup>+</sup> PD-1<sup>+</sup> T<sub>FH</sub>-like cells. (d) Percentages of IL-21 producing CD4<sup>+</sup>CXCR5<sup>+</sup> PD-1<sup>+</sup> T<sub>FH</sub>-like cells according to the presence of autoantibodies in patients group. Each data point represents an individual subject, horizontal lines show the mean values with SD. Statistically significant differences are indicated by \*, p < 0.05; \*\*, p < 0.01; \*\*\*, p < 0.001.

**Figure 3.** Circulating T<sub>FH</sub>-like cells are associated with the expansion of IL-21 producing T<sub>FH</sub>-like cells. Correlation between the percentages of CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>+</sup>PD-1<sup>+</sup> T<sub>FH</sub>-like cells and CD4<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>+</sup>IL-21<sup>+</sup> T<sub>FH</sub>-like cells in patients with pSS (black dots with solid line) and SLE (clear dots with dashed line).

**Figure 4.** Quantification of peripheral IL-10 producing B10 and B10<sub>PRO</sub> cells in pSS patients, SLE patients and healthy controls. PBMCs were isolated from 25 pSS patients, 25 SLE patients and 21 healthy controls, then to gain B10 cell frequencies, cells were incubated with CpG+PIB for 5 h. B10+B10<sub>PRO</sub> cell maturation was induced with CpG stimulation for 48 h with PIB added during the last 5 h. Regarding the exact assessment



of IL-10 production, we used BFA and PIB alone controls during the procedure. Representative dot plots show IL-10 producing B cells in response to different conditions. (a) Percentages of IL-10<sup>+</sup>CD19<sup>+</sup> B10 cells. (b) Percentages of IL-10<sup>+</sup>CD19<sup>+</sup> B10 and B10<sub>PRO</sub> cells in pSS patients group (n=25), SLE patient group (n=25) and control group (n=21) in response to BFA, PIB or CpG+PIB. Results are presented as box and whiskers plot, horizontal lines show the mean values. Statistically significant differences are indicated by \*, p < 0.05; \*\*, p < 0.01; ns, no significant differences between patient and control groups within an experiment. Statistically significant differences are indicated by #, p<0.0001 between PIB alone and PIB+CpG stimulation within the groups. PIB: Phorbol 12-myristate 13-acetate [PMA] + Ionomycin + Brefeldin A

**Figure 5.** Correlation analysis between peripheral T<sub>FH</sub>-like cells and transitional B cell subpopulations. (a) Correlation between the percentages of T<sub>FH</sub>-like cells and CD19<sup>+</sup>CD38<sup>hi</sup>CD24<sup>hi</sup>CD27<sup>-</sup> transitional B cells in pSS patients with EGMs. (b) Correlation between the percentages of IL-21 producing T<sub>FH</sub>-like cells and transitional B cells in pSS patients with EGMs. (c) Correlation between the proportions of IL-21 producing T<sub>FH</sub>-like cells and CD19<sup>+</sup>CD38<sup>+</sup>CD24<sup>+</sup> mature-naïve B cells in pSS patients with EGMs. (d) Correlation between the ratios of T<sub>FH</sub>-like cells and CD19<sup>+</sup>IgD<sup>-</sup>CD27<sup>-</sup> DN B cells in SLE patients with SLEDAI<6. (e) Correlation between the frequencies of T<sub>FH</sub>-like cells and CD19<sup>+</sup>CD38<sup>-</sup>CD24<sup>hi</sup>CD27<sup>+</sup> primarily memory B cells in SLE patients with SLEDAI<6. (f) Correlation between the percentages of IL-21 producing CD4<sup>+</sup>CXCR5<sup>+</sup> T cells and CD19<sup>+</sup>CD38<sup>hi</sup>CD27<sup>hi</sup> plasmablasts in the whole SLE patients group. Each data point represents an individual subject.

**Figure 6.** Summarized model showing the outcome of disturbed tolerance during B cell development in pSS and SLE. In physiological circumstances, to avoid autoreactivity, during B cell development multiple tolerance checkpoints are applied for the selection of autoreactive B cells<sup>43</sup>. Abnormalities in checkpoint regulation, along with hyperactivation of T<sub>FH</sub> cells and self-reactive B cells contribute to the development of autoimmunity in susceptible individuals. Breaking of early B-cell tolerance leads to consequential accumulation of transitional and naive B cells in the periphery. Perturbation of late peripheral tolerance in secondary lymphoid organs allows autoreactive mature B cells to undergo extrafollicular or germinal centre responses, which are supported by T<sub>FH</sub> cells, and differentiate into memory B cells or plasmablasts<sup>15, 44, 45</sup>. Derailed B cell homeostasis and increased T<sub>FH</sub> cells responses manifest in characteristic changes in B cell distribution and accumulation of circulating T<sub>FH</sub>-like cell in pSS and SLE.

**Figure S1.** Viability of the CpG exposed cells were determined by 7-aminoactinomycin-D (7-AAD) staining using flow cytometric analysis. (a) Representative dot plots show the 7-AAD<sup>+</sup> dead cells and 7-AAD<sup>-</sup> viable cells in the PBMC. Underneath, smaller dot plots indicate the differences in the presence of B cells among the viable or dead cell populations. (b) Representative dot plots show the frequency of CD19<sup>+</sup>7-AAD<sup>+</sup> cells within the lymphocytes. (c) Percentages of 7-AAD<sup>+</sup> (left) and CD19<sup>+</sup>7-AAD<sup>+</sup> cells (right) in pSS patients group (n=5), SLE patient group (n=7) and control group (n=4) in response to BFA, PIB or CpG+PIB.

**Figure S2.** Evaluation of T<sub>FH</sub> markers on naive CD45RA<sup>+</sup> T cells and active or memory CD45RA<sup>-</sup> T cells in pSS patients, SLE patients and healthy subjects. PBMCs were isolated from 9 pSS patients, 8 SLE patients and 6 healthy controls then were stained as described previously. Representative dot plots show the gating strategy for the determination of CD4<sup>+</sup>CD45RA<sup>-</sup> and CD4<sup>+</sup>CD45RA<sup>+</sup> subsets. (a) Percentages of CXCR5<sup>+</sup>ICOS<sup>+</sup> in the CD4<sup>+</sup>CD45RA<sup>-</sup> (left) and in the CD4<sup>+</sup>CD45RA<sup>+</sup> (right) subsets. (b) Percentages of CXCR5<sup>+</sup>PD-1<sup>+</sup> in the CD4<sup>+</sup>CD45RA<sup>-</sup> (left) and in the CD4<sup>+</sup>CD45RA<sup>+</sup> (right) subsets. (c) Association between the ratio of CXCR5<sup>+</sup>ICOS<sup>+</sup> cells and CXCR5<sup>+</sup>PD-1<sup>+</sup> cells in the CD4<sup>+</sup>CD45RA<sup>-</sup> subset. Each data point represents an individual subject, horizontal lines show the mean values with SD.

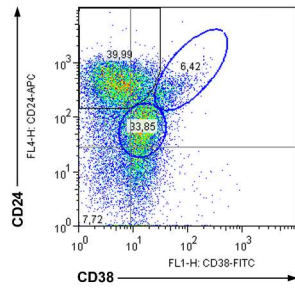
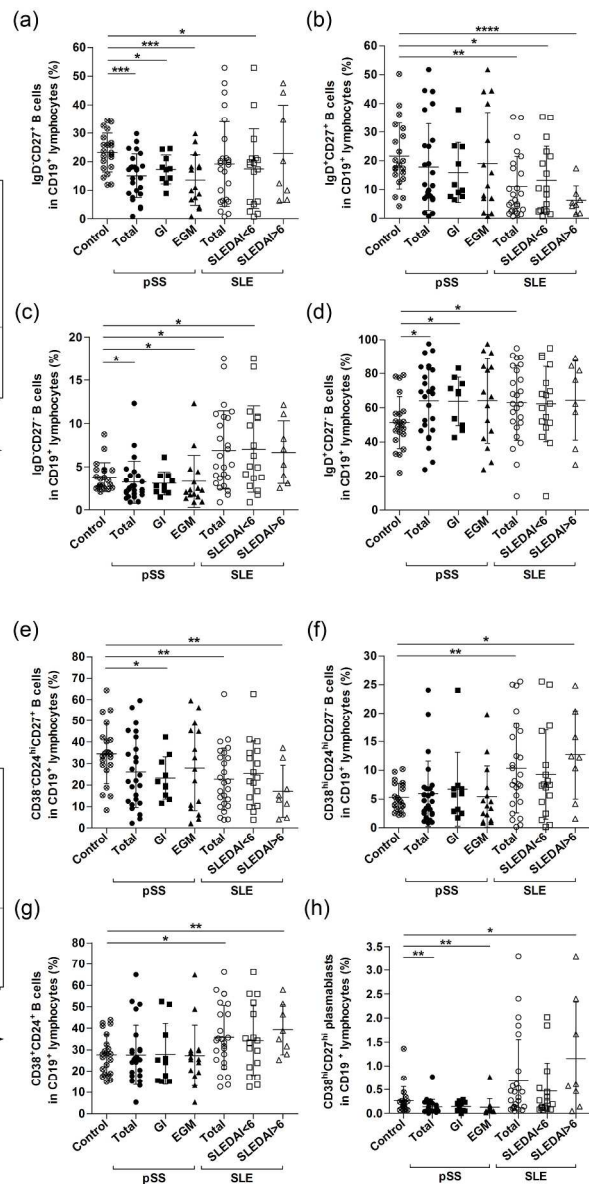
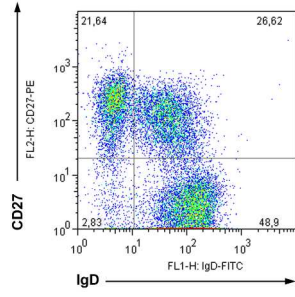
## Tables

Table 1. Association of certain T<sub>FH</sub>-like cell proportions with serological parameters

Association	Disease	Variable	Correlation coefficient	p-value
Positive	pSS	CD4 <sup>+</sup> CXCR5 <sup>+</sup> ICOS <sup>+</sup> PD-1 <sup>+</sup> T <sub>FH</sub> -like cells vs. levels of serum IgG antibody	Spearman R=0.6143	0.0018 **
		CD4 <sup>+</sup> CXCR5 <sup>+</sup> ICOS <sup>+</sup> PD-1 <sup>+</sup> T <sub>FH</sub> -like cells vs. levels of serum RF	Spearman R=0.6219	0.0101 *
		CD4 <sup>+</sup> CXCR5 <sup>+</sup> PD-1 <sup>+</sup> IL-21 <sup>+</sup> T <sub>FH</sub> cells vs. levels of serum IC	Pearson R=0.5734	0.0128 *
	SLE	CD4 <sup>+</sup> CXCR5 <sup>+</sup> IL-21 <sup>+</sup> T <sub>FH</sub> cells vs. levels of serum IC	Spearman R=0.4723	0.0478 *

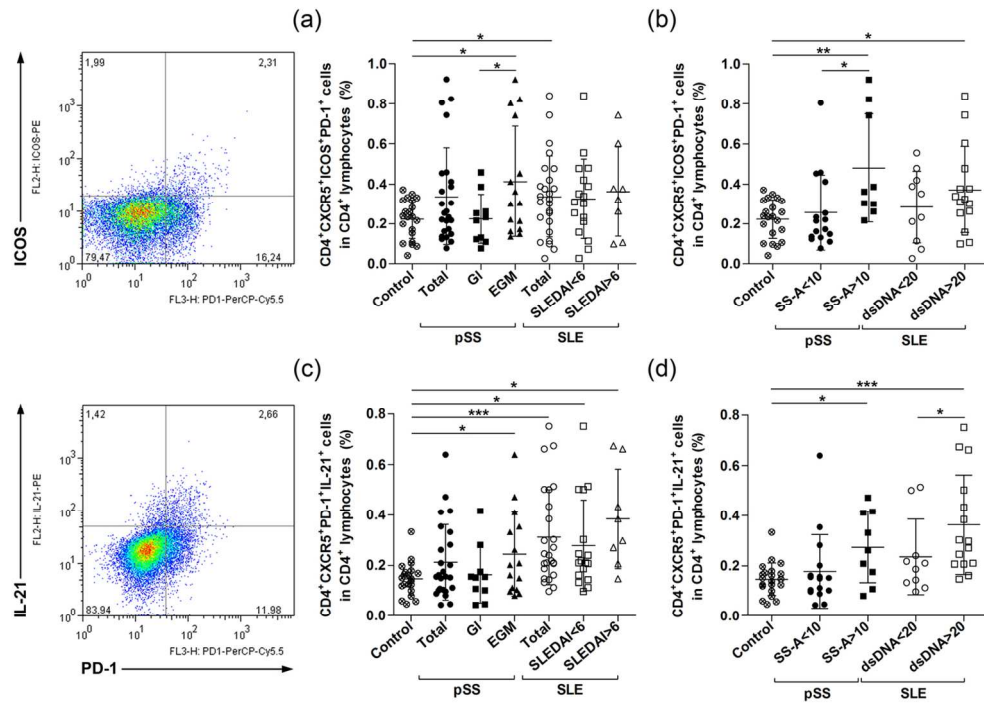
Table 2. Association of certain B cell proportions with laboratory parameters

Association	Disease	Variable	Correlation coefficient	p-value
Positive	pSS	CD19 <sup>+</sup> CD38 <sup>hi</sup> CD24 <sup>hi</sup> CD27 <sup>-</sup> transitional B cells vs. levels of serum IgG	Spearman R=0.4884	0.0181 *
		CD19 <sup>+</sup> CD38 <sup>hi</sup> CD27 <sup>hi</sup> plasmablasts vs. levels of serum IC	Spearman R=0.4907	0.0387 *
	SLE	CD19 <sup>+</sup> CD38 <sup>hi</sup> CD27 <sup>hi</sup> plasmablasts vs. levels of serum anti-dsDNA	Spearman R=0.5320	0.0090 **
Negative	SLE	CD19 <sup>+</sup> CD38 <sup>hi</sup> CD24 <sup>hi</sup> CD27 <sup>-</sup> transitional B cells vs. levels of serum C3	Pearson R=-0.4914	0.0172 *



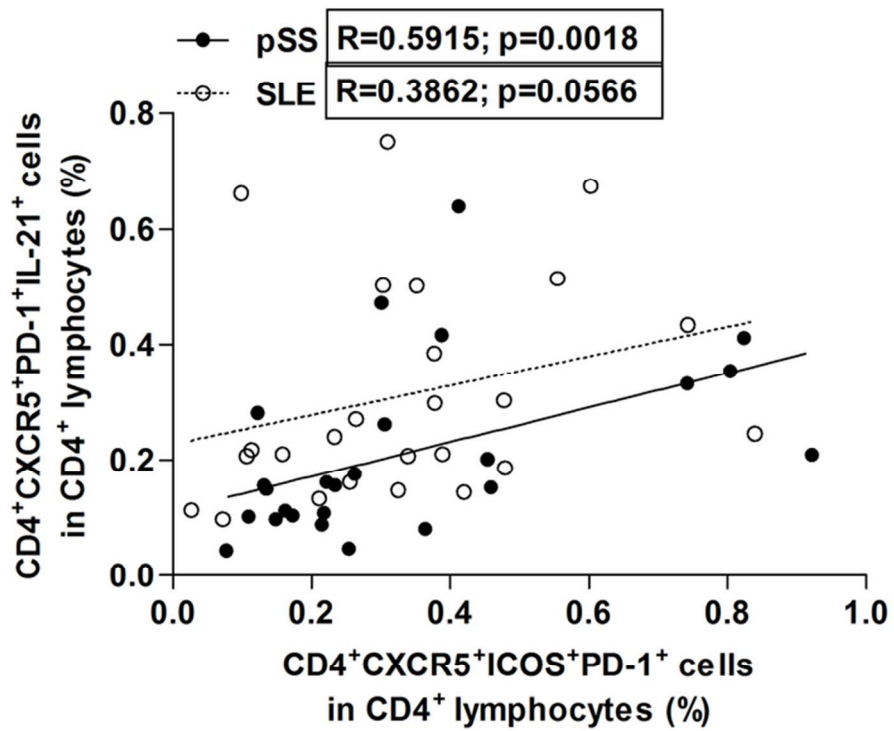
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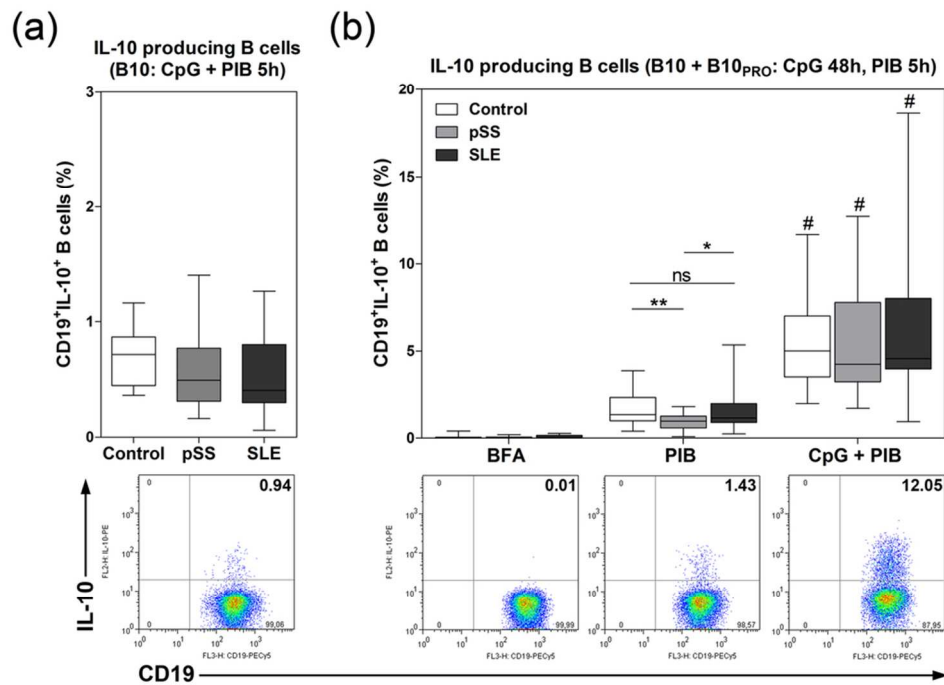
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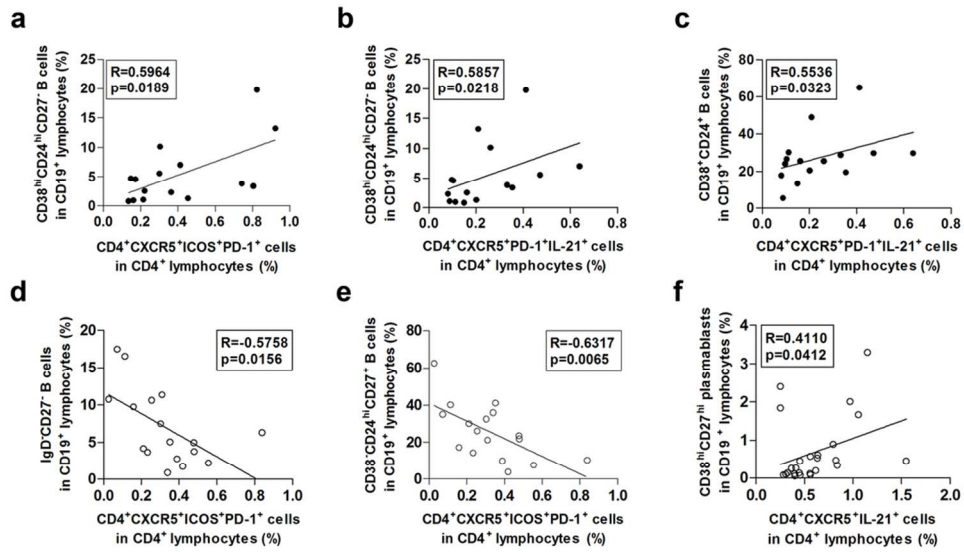
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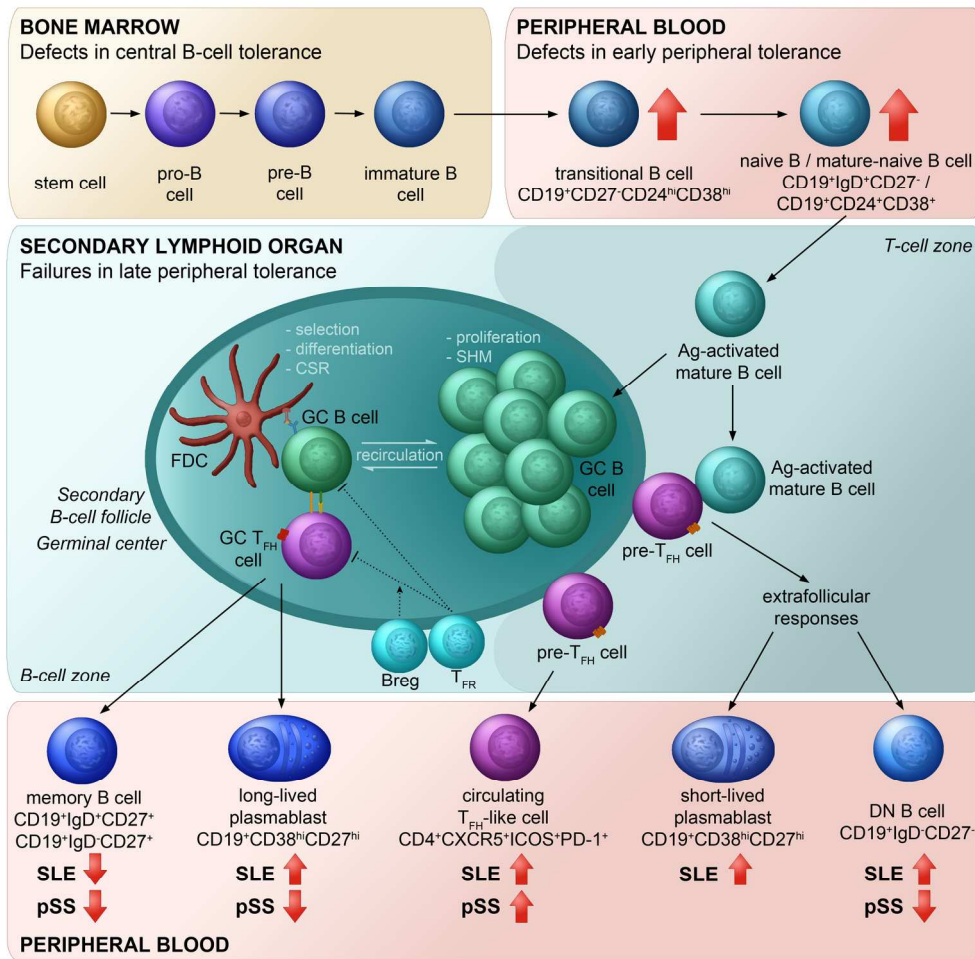
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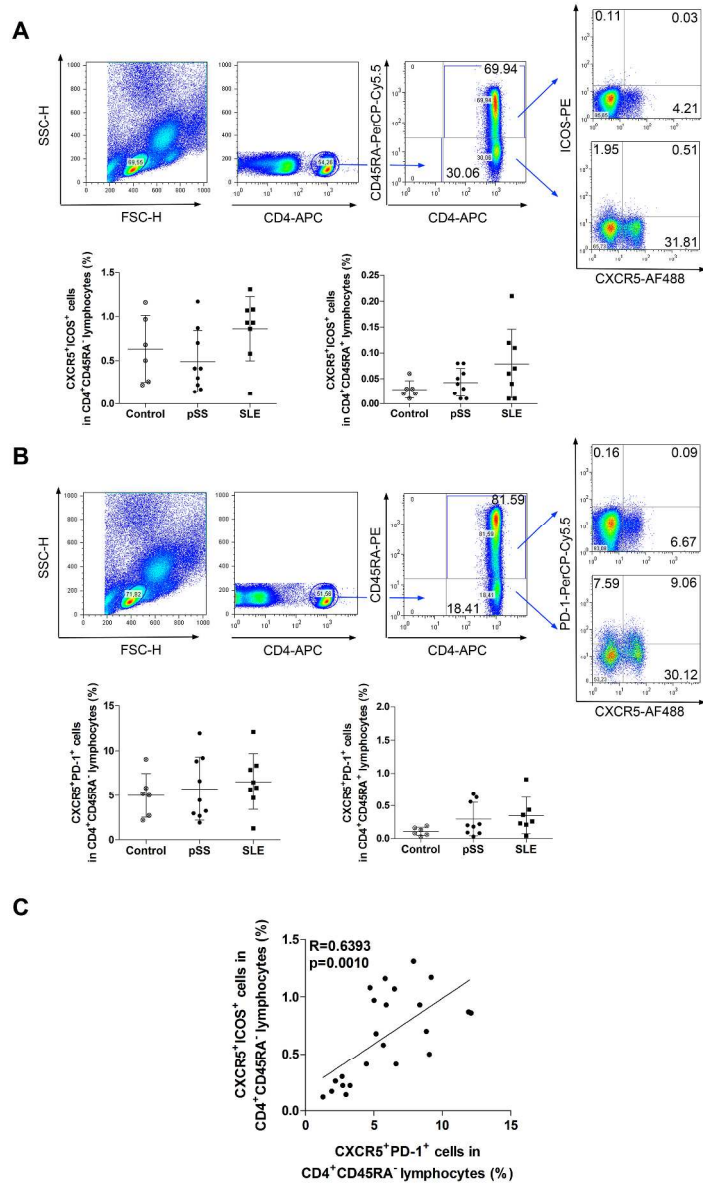
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