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ORIGINAL ARTICLE

Lymphocyte subpopulations in Sjögren's syndrome are distinct in anti-SSA-positive patients and related to disease activity

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Abstract

Objectives Sjögren's syndrome (SjS) patients exhibit great phenotypical heterogeneity, reinforced by the positiveness of anti-SSA antibody. We aimed to evaluate lymphocyte subpopulations in SSA-positive (SSA+SjS) and SSA-negative (SSA−SjS) SjS patients, *Sicca* patients, and healthy controls (HC), and to investigate associations between lymphocyte subpopulations and disease activity in SjS.

Methods According to the fulfilment of the ACR/EULAR 2016 classification criteria, patients were included as SjS or as *Sicca*. HC were selected from the Ophthalmology outpatient clinic. Lymphocyte subpopulations were characterized by flow cytometry. Statistical analysis was performed with GraphPad Prism^{TM}, with statistical significance concluded if $p < 0.05$.

Results We included 53 SjS patients (38 SSA+ and 15 SSA−), 72 *Sicca*, and 24 HC. SSA+SjS patients presented increased IL-21^{+}CD4^{+} and CD8^{+} T cells compared to *Sicca* and HC, whereas compared to SSA−SjS patients, only IL-21^{+}CD4^{+} T cell percentages were increased and Tfh17 percentages and numbers were decreased. Compared to *Sicca* and HC, SSA+SjS patients had higher levels of CD24^{Hi}CD38^{Hi} B cells, naïve B cells, and IgM$^{-/+}$CD38^{++} plasmablasts, and lower levels of memory B cells, including CD24^{Hi}CD27^{+} B cells. SSA+SjS patients with clinically active disease had positive correlations between ESSDAI and IL-21^{+}CD4^{+} ($p = 0.038$, $r = 0.456$) and IL-21^{+}CD8^{+} T cells ($p = 0.046$, $r = 0.451$).

Conclusions In SjS, a distinct lymphocyte subset distribution profile seems to be associated with positive anti-SSA. Moreover, the association between ESSDAI and IL-21^{+}CD4^{+} and IL-21^{+}CD8^{+} (follicular) T cells in SSA+SjS patients suggests the

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involvement of these cells in disease pathogenesis and activity, and possibly their utility for the prognosis and assessment of response to therapy.

Key Points
- *SSA+SjS patients have a pronounced naïve/memory B cell imbalance.*
- *SSA+SjS patients have more active disease associated with IL-21$^+$CD4$^+$ and IL-21$^+$CD8$^+$ follicular T cell expansion.*
- *IL-21$^+$CD4$^+$ and IL-21$^+$CD8$^+$ T cell quantification may be useful for the prognosis and assessment of response to therapy.*

Keywords B lymphocytes · Disease activity · Sjögren's syndrome · SSA · T lymphocytes

Background

Sjögren's syndrome (SjS) is a chronic immune-mediated disease characterized by lymphocytic infiltration and lesion of exocrine glands, with a variable clinical spectrum, from isolated mucosal dryness to systemic involvement with extraglandular manifestations [1].

The 2016 American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) classification criteria [2] demand either anti-SSA antibodies or a positive minor salivary gland (SG) biopsy (highly specific but invasive and dependent on standardization).

SjS's immunopathogenesis is complex and involves the innate and adaptive immune system. B cell hyperactivity is the hallmark of SjS, playing a major role in pathogenesis and clinical evolution [3]. Interactions between T cells and activated B cells occur in germinal center (GC)–like structures within target organs, such as SG and lachrymal glands, which become dysfunctional [4]. Additionally, T cells infiltrate target organs and produce pro-inflammatory cytokines resulting in B cell activation [5].

Follicular helper T cells (Tfh) have recently emerged as a key player in SjS pathogenesis. Tfh cells are a major source of IL-21, a cytokine that mediates B cell differentiation and proliferation and promotes the ectopic formation of GC-like structures [6]. The chemokine receptor X5 (CXCR5) induces the homing of follicular T cells to lymph nodes, particularly to B cell sites, and is a surface marker useful in the identification of follicular-related T cells. CXCR5 is also expressed in CD8$^+$ T cells, suggesting the existence of a follicular-related cytotoxic (CD8$^+$) T cell subset (Tfc) [7]. Tfh differentiation is promoted in the SG of SjS patients, who seem to present increased circulating Tfh cells [8].

Although the frequency of regulatory T cells (Tregs) in SjS is variable in the literature, SjS patients seem to present Tregs deficiencies [9], contributing to disease pathogenesis.

Regarding B cells, increased naïve B cells and decreased memory B cells, particularly unswitched memory, are typical in SjS patients [10]. Some B cell subsets, known as regulatory B cells (Bregs), contribute to the regulation of immune responses, mainly through the secretion of interleukin-10 (IL-10). Bregs suppress effector T cells, inhibit Tfh differentiation, and induce Tregs [11]. Recently, CD19$^+$CD24$^+$CD38^{hi} Bregs showed reduced IL-10 production and defective Tfh inhibitory capacity in SjS patients [12].

The presence of anti-SSA antibodies is associated to distinct phenotypic features of SjS [13]. We sought to investigate whether lymphocyte subpopulations were distinctive in SSA+ SjS patients versus SSA−SjS, *Sicca* patients, and healthy controls (HC), and their impact in disease activity.

Methods

Population

We enrolled patients with confirmed or suspected SjS, who were consecutively recruited and classified as SjS if ACR/EULAR 2016 classification criteria were fulfilled, or otherwise included in the *Sicca* group. All patients had been subjected to a multidisciplinary evaluation which included ocular evaluation (Schirmer's test and corneal staining score), and unstimulated salivary flow assessment (Table 1). Secondary SjS patients were excluded.

The HC group consisted of women without *Sicca* symptoms, selected from the Ophthalmology outpatient clinic.

Disease activity in SjS was assessed with the EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI) [14]. Patients were considered having clinically active disease if they scored in any ESSDAI domain except for the hematologic and biologic ones. Patients were stratified according to disease activity as low (ESSDAI < 5) or moderate/high (ESSDAI ≥ 5) [15].

Informed consent was obtained from all participants. This study was approved by the Ethics committees of *Hospital CUF Descobertas*, *Instituto Português de Reumatologia* and NOVA Medical School (no 17/2016/CEFCM).

Flow cytometry measurements

Blood samples were collected into EDTA-containing tubes, processed, and analyzed within 24 h after collection. Pre-

Table 1 Patients' characteristics

	Sjögren's syndrome (SjS)				Group's comparison (*p* value)		
	Total SjS *n* = 53	SSA+SjS *n* = 38	SSA−SjS *n* = 15	Sicca *n* = 72	Total SjS vs Sicca	SSA+SjS vs SSA−SjS	SSA+SjS vs Sicca
Sex, F/M	53/0	38/0	15/0	71/1	1.000	1.000	1.000
Age at onset (y), mean (SD)	46.2 (11.6)	44.4 (11.1)	50.7 (11.8)	50.8 (10.0)	*0.022*#	0.092#	*0.004*#
Age (y), mean (SD)	57.5 (11.9)	56.8 (12.4)	60.0 (9.0)	60.8 (11.0)	0.127#	0.538#	0.109#
Age at diagnosis (y), mean (SD)	51.7 (11.9)	49.6 (12.0)	57.0 (10.2)	57.4 (10.4)	*0.006*#	*0.032*#	*0.001*#
Symptom duration (y), mean (SD)	11.5 (7.6)	12.3 (7.6)	9.3 (7.3)	10.0 (5.4)	0.509#	0.183#	0.174#
Ocular symptoms, *n* (%)	50 (94.3)	36 (94.7)	14 (93.3)	69 (95.8)	0.650	1.000	0.610
Oral symptoms, *n* (%)	51 (96.2)	36 (94.7)	15 (100.0)	69 (95.8)	1.000	1.000	0.610
Low Schirmer's/keratitis *sicca*, *n* (%)	33 (62.3)	22 (57.9)	11 (73.3)	39 (54.2)	0.464	0.359	0.840
Decreased salivary flow, *n* (%)	41 (77.4)	30 (78.9)	11 (73.3)	50 (69.4)	0.417	0.722	0.370
Clinically active disease, *n* (%)	26 (49.1)	21 (55.3)	5 (33.3)	10 (13.9)	*< 0.001*	0.224	*< 0.001*
ESSDAI, mean (SD) [min–max]	2.5 (3.0) [0–14]	3.0 (3.2) [0–14]	1.3 (1.9) [0–7]	NA	-	*0.025*#	-
ESSDAI ≥ 5, *n* (%)	9 (17.0)	8 (21.1)	1 (6.7)	NA	-	0.418#	-
Salivary gland swelling, *n* (%)	8 (15.1)	8 (21.1)	0 (0)	2 (2.8)	*0.018*	0.088	*0.003*
Joint symptoms, *n* (%)	23 (43.4)	18 (47.4)	5 (33.3)	32 (50.8)	1.000	0.539	0.842
Skin symptoms, *n* (%)	18 (34.0)	13 (34.2)	5 (33.3)	16 (22.2)	0.160	1.000	0.182
Other extra-glandular involvement, *n* (%)	9 (17.0)	8 (21.1)	1 (6.7)	1 (1.4)	*0.002*	0.418	*0.001*
Raynaud's phenomenon, *n* (%)	7 (13.2)	7 (18.4)	0 (0.0)	18 (25.0)	0.118	0.172	0.483
Focus score ≥ 1, *n* (%)	39 (76.5)[a]	24 (66.7)[c]	15 (100)	3 (4.2)	*< 0.001*	*0.011*	*< 0.001*
SSA, *n* (%)	38 (71.7)	38 (100)	0 (0.0)	1 (1.4)	*< 0.001*	*< 0.001*	*< 0.001*
SSB, *n* (%)	19 (39.6)[b]	19 (57.6)[d]	0 (0.0)	1 (1.4)	*< 0.001*	*< 0.001*	*< 0.001*
ANA ≥ 1/320, *n* (%)	43 (81.1)	33 (86.8)	10 (66.7)	39 (54.2)	*0.002*	0.124	*0.001*
ANA ≥ 1/640, *n* (%)	31 (58.5)	28 (73.7)	3 (20.0)	26 (36.1)	*0.019*	*0.001*	*< 0.001*
RF positive, *n* (%)	31 (58.5)	26 (68.4)	5 (33.3)	19 (26.4)[e]	*0.001*	*0.030*	*< 0.001*
Gammaglobulin ≥ 1.6 g/dl, *n* (%)	14 (26.4)	13 (34.2)	1 (6.7)	3 (4.2)[e]	*0.001*	0.080	*< 0.001*
Therapy (any), *n* (%)	30 (56.6)	21 (55.3)	9 (60.0)	32 (44.4)	0.207	1.000	0.319
Glucocorticoids, *n* (%)	18 (34.0)	14 (36.8)	4 (26.7)	18 (25.0)	0.320	0.539	0.270
Hydroxychloroquine, *n* (%)	19 (35.8)	13 (34.2)	6 (40.0)	21 (29.2)	0.445	0.756	0.666
Immunosuppressants, *n* (%)	9 (17.0)	6 (15.8)	3 (20.0)	9 (12.5)	0.608	0.701	0.771

Patient's characteristics are represented as number of occurrences (*n*) and percentages (%). Whenever there were missing values, percentages reflect the number of occurrences over the number of patients tested for the item ([a] *n* = 51; [b] *n* = 48; [c] *n* = 36; [d] *n* = 33; [e] *n* = 71). Joint symptoms include arthritis and joint pain of inflammatory origin, but only cases that would score in the articular domain of ESSDAI were considered extra-glandular disease. Likewise, in some patients, skin involvement (which not included xerosis) was not considered extra-glandular disease if it would not score in the cutaneous domain of ESSDAI. Statistically significant results are indicated in italic

SjS, primary Sjögren's syndrome; *F*, female; *M*, male; *y*, years; *SSA/SSB*, Sjögren's syndrome A/B antibody; *ANA*, antinuclear antibody; *RF*, rheumatoid factor; *ESSDAI,* European Sjögren's syndrome disease activity index

*Fisher's exact test

Mann-Whitney *U* test

validated panels of monoclonal antibodies were used to characterize lymphocyte subpopulations, including anti-CD3^{A}, anti-CD4^{A}, anti-CD19^{B}, anti-CD24^{B}, anti-CD25^{B}, anti-CD27^{B}, anti-CD38^{B}, anti-CD127^{B}, anti-CCR6^{A}, anti-CCR7^{B}, anti-CXCR3^{A}, anti-CXCR5^{A}, anti-IgD^{B}, and anti-IgM^{A} (^{A}BD Biosciences; ^{B}Biolegend), as described elsewhere [16].

Samples were acquired in a 4-color BD FACS Calibur™ cytometer (BD Biosciences) and analyzed with Cell Quest Pro™ software (BD Biosciences). This study addressed several subsets of T cells, including Tregs and CXCR5^{+} follicular-related T cells, as well as naïve and memory B cell subsets.

Each subset was evaluated in percentages and absolute counts, using BD Trucount tubes™ (BD Biosciences).

Complete gating strategies are described in Fig. 1.

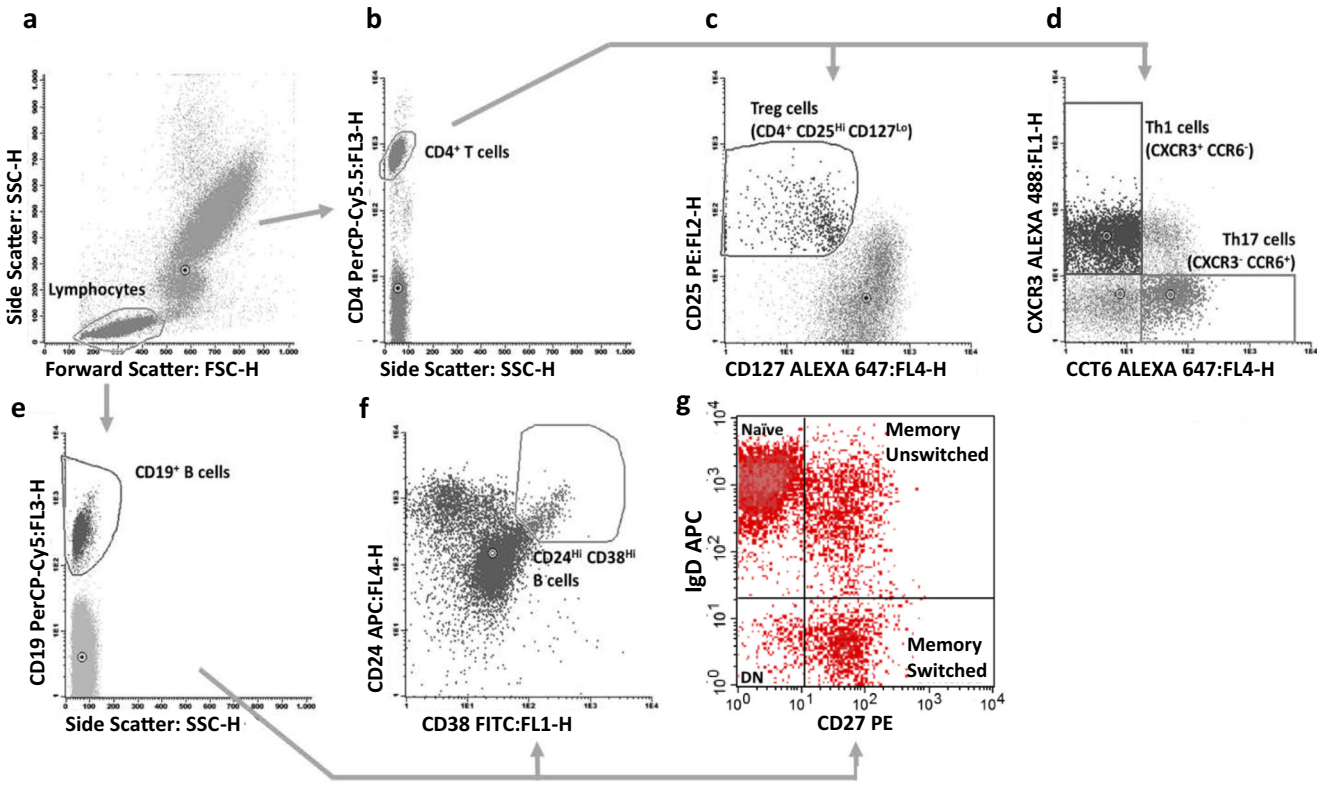

Fig. 1 Gating strategy for the identification of distinct T and B cell subsets. (a) Lymphocytes were identified according to forward and side scatter characteristics. (b and e) $CD4^+$ T and B cells were identified as $CD4^+$ (b) and $CD19^+$ (e) cells within the lymphocyte gate, respectively. (c) Treg subset was identified according to the expression of CD25 and CD127 within the $CD4^+$ T cells gate. (d) T-helper (Th) subsets were identified according to the expression of CXCR3 and CCR6 in Th1 ($CXCR3^+CCR6^-$) and Th17 ($CXCR3^-CCR6^+$) cells. (f) B cells with a regulatory phenotype were identified as $CD24^{Hi}CD38^{Hi}$ cells within the B lymphocyte gate. (g) B cell subsets were identified according to the expression of IgD and CD27 in naive, IgD^+CD27^-; unswitched memory/marginal zone like (MZ), IgD^+CD27^+; switched memory, IgD^-CD27^+; and double-negative (DN) memory B cells, IgD^-CD27^-

Functional assays for the evaluation of IL-21-producing CD4 and CD8 T cells

To characterize IL-21 and IL-17-producing T cells, heparinized blood was collected and stimulated with PMA (phorbol myristate acetate; 50 ng/mL) and ionomycin (1 μg/mL), for 5 h at 37 °C in a 5% CO_2 atmosphere, in the presence of brefeldin A. After stimulation, cells were lysed, washed, and then incubated with anti-CD3 and anti-CD8 for surface staining. Afterwards, cells were treated according to the manufacturer's instructions for the BD Fixation/Permeabilization Solution Kit with BD GolgiPlug™ (BD Biosciences) and marked with anti-IL-21 and anti-IL17. Stimulated and unstimulated tubes were run in parallel to assure proper stimulation and staining controls. Gating strategies are presented in Fig. 2.

Statistical analysis

GraphPad Prism™ software 8.3.0 for Windows was used for statistical analysis. Categorical variables were expressed as numbers and percentages and analyzed using Fisher's exact test. Normality was assessed using the D'Agostino and Pearson test, and continuous variables were presented as means (standard deviation) or medians (25th–75th percentile), as applicable. The Mann-Whitney *U* test was used to compare every 2-independent groups. Spearman correlation coefficients were calculated to assess correlations. Statistical significance was concluded when $p < 0.05$.

Results

Patient's characteristics

Fifty-three SjS and 72 *Sicca* patients were included. Thirty-eight SjS patients (71.7%) presented anti-SSA antibodies (SSA+SjS) while 15 (28.3%) were anti-SSA-negative (SSA–SjS). The HC group included 24 women. Table 1 describes the demographic, clinical, and immunological characteristics of patients.

SSA+SjS had a younger age of onset (44.4 years vs 50.7 years; $p = 0.092$) and were diagnosed earlier (49.7 years vs 57.0 years; $p = 0.032$) than SSA−SjS. None of the SSA−SjS

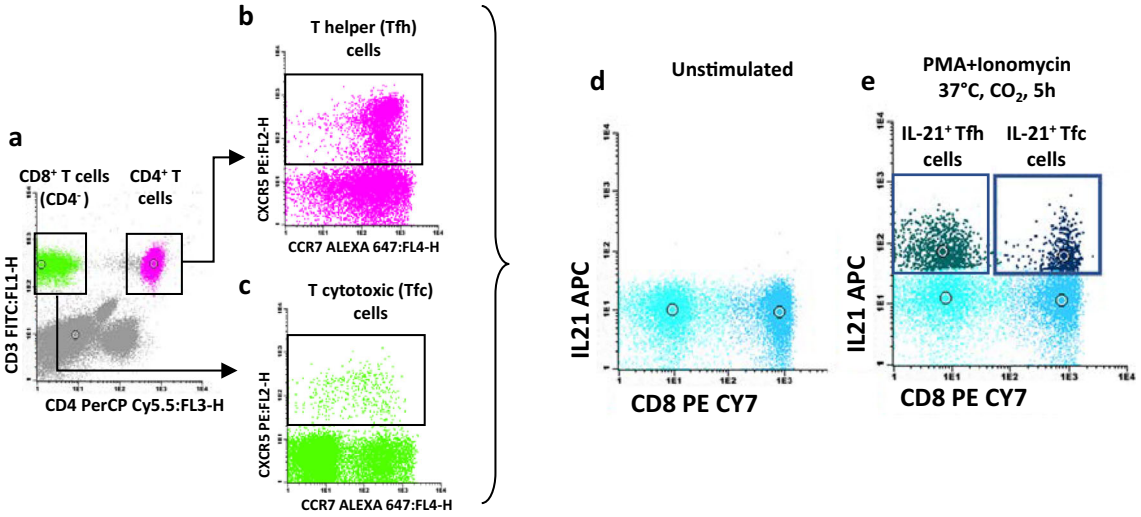

Fig. 2 Identification of follicular T cell subsets. (a–c) Gating strategy for the identification of follicular-related ($CXCR5^+CCD7^{-/+}$) cytotoxic (b) and helper (c) T cells. (d and e) IL-21-producing $CXCR5^+$ $CD4^+$ and $CD8^+$ T cells were analyzed for the expression of IL-21 after a 5-h incubation period without stimulation (d) and after stimulation (e) with PMA (phorbol myristate acetate) and ionomycin, for 5 h at 37 °C in a 5% CO_2 atmosphere in the presence of brefeldin A. Unstimulated tubes were used to assess IL-21 positivity

patients presented parotid enlargement or Raynaud's phenomenon, although significance was not reached compared to SSA+SjS. Clinically active disease was present in 55.3% of SSA+SjS patients, with a mean ESSDAI of 3.0 (3.2), and in 33.3% of SSA−SjS with a mean ESSDAI of 1.3 (1.9) (p = 0.025). Rheumatoid factor (RF) was positive in 68.4% of SSA+SjS, significantly more frequent than in SSA−SjS (33.3%, p = 0.030) and *Sicca* (26.4%, $p < 0.001$). Hypergammaglobulinemia was also more frequent in SSA+SjS (34.2%) compared to SSA−SjS (6.7%, p = 0.080) and *Sicca* (4.2%, $p < 0.001$).

Lymphocyte characterization

Complete results are presented in Table 2 and Supplementary Table 1.

$CD4^+$ and $CD8^+$ T cell subsets

Compared to both *Sicca* and HC, SjS patients presented lower $CD4^+$ T cell counts, especially when considering the SSA+SjS subgroup (percentages, $p \leq 0.032$; absolute counts, $p \leq 0.046$). $CD4^+$ T cell counts were also significantly lower in *Sicca* compared to HC ($p < 0.001$). Both SjS groups presented higher $CD8^+$ T cell percentages compared to *Sicca* and HC, with no differences in counts.

Treg counts were lower in all patient groups when compared to HC ($p \leq 0.042$), although with higher percentages in SSA+SjS compared to *Sicca* (p = 0.007) (Fig. 3). When considering only SSA+SjS patients with moderate/high disease activity (ESSDAI ≥ 5, n = 8), a negative correlation was found between Treg counts and the ESSDAI (p = 0.011, r = − 0.854) (Fig. 4).

Percentages of $CXCR5^+$ $CD4^+$ or $CD8^+$ T cells were similar in all patient groups. Nevertheless, lower levels of $CXCR5^+$ T-helper cell counts were observed in SSA+SjS compared to *Sicca* (p = 0.026), and in all patient groups compared to HC ($p \leq 0.022$).

Regarding $CXCR5^+$Th subsets, $CXCR5^+$Th1 ($CCR3^+CCR6^-$) percentages were higher in SSA+SjS than in *Sicca* (p = 0.003) and HC (p = 0.012), while $CXCR5^+$Th17 ($CCR3^-CCR6^+$) percentages were lower in SSA+SjS compared to SSA−SjS (p = 0.036) and *Sicca* (p = 0.024). However, $CXCR5^+$Th1 counts were lower in SSA+SjS and *Sicca* compared to HC ($p \leq 0.001$), whereas SSA+SjS presented lower $CXCR5^+$Th17 counts compared to all groups ($p \leq 0.015$) (Fig. 3). To investigate possible relations with disease activity, we further explored the association between Tfh-related subsets and the ESSDAI (Fig. 4). SSA+SjS patients showed a trend for a positive correlation between ESSDAI and $CXCR5^+$Th1 percentages (r = 0.294, p = 0.073), which was statistically significant in patients with clinically active disease (r = 0.616, p = 0.003), and among those, even stronger when considering patients with moderate/high disease activity (r = 0.927, p = 0.002). Contrarily, ESSDAI and $CXCR5^+$Th17 percentages (r = − 0.341, p = 0.036) showed a negative correlation, also slightly stronger in patients with active disease (r = − 0.456, p = 0.038). Despite the low number of patients with moderate/high disease activity, a similar trend was also observed for ESSDAI and $CXCR5^+$Th17 percentages (r = − 0.659, p = 0.088) in this group. No correlations were found between Tfh-related subsets and ESSDAI in SSA−SjS patients.

Table 2 Comparison of T and B cell subsets' percentages in SjS, Sicca, and healthy control groups

	Sjögren's syndrome			Sicca	HC	Group's comparisons (*p* values)					
	Total SjS *n* = 53	SSA+SjS *n* = 38	SSA−SjS *n* = 15	*n* = 72	*n* = 24	SSA+ vs SSA−	SSA+ vs Sicca	SSA+ vs HC	SSA− vs Sicca	SSA− vs HC	Sicca vs HC
Lymphocytes (within leukocytes)	31.5 (23.4–38.1)	31.7 (23.9–36.4)	31.5 (22.3–40.2)	32.2 (25.7–37.1)	36.7 (29.3–39.8)	0.499	0.531	*0.034*	0.900	0.242	*0.027*
B cells	9.7 (6.9–13.3)	9.8 (5.9–16.0)	9.7 (7.0–11.0)	11.7 (8.7–14.8)	10.4 (8.6–13.7)	0.636	0.319	0.723	*0.023*	0.231	0.392
T cells	74.8 (67.3–78.5)	74.9 (67.3–78.5)	74.6 (70.5–78.8)	73.1 (67.6–77.3)	74.7 (70.1–79.6)	0.721	0.409	0.391	0.266	0.836	0.059
T cell subsets											
$CD4^+$	61.2 (53.1–66.6)	61.2 (51.2–66.9)	59.7 (55.1–65.1)	66.0 (60.0–72.6)	69.1 (60.8–75.9)	0.897	*0.004*	*0.001*	*0.032*	*0.006*	0.227
Th1	40.3 (28.6–46.0)	38.1 (27.2–45.9)	41.2 (32.1–46.3)	38.6 (30.2–47.4)	35.7 (31.7–45.9)	0.383	0.535	0.878	0.620	0.464	0.625
Th17	19.5 (13.9–28.6)	18.0 (13.8–27.2)	26.6 (19.2–31.6)	22.7 (16.4–28.7)	23.1 (17.0–31.7)	0.154	0.085	0.089	0.603	0.916	0.630
Tregs	8.2 (6.5–10.4)	8.6 (7.1–10.6)	7.1 (6.0–8.5)	7.6 (6.7–8.9)	7.4 (6.7–8.8)	0.085	*0.007*	0.067	0.464	0.535	0.948
$CD8^+$	38.8 (33.0–46.9)	38.6 (32.5–48.9)	40.4 (34.9–44.9)	34.1 (27.4–39.7)	30.9 (23.4–39.2)	0.911	*0.010*	*0.004*	*0.025*	*0.007*	0.231
$CXCR5^+CD4^+$ T cells (within $CD4^+$ T cells)	18.4 (14.7–23.6)	18.5 (14.2–23.0)	16.9 (15.3–24.7)	18.3 (14.9–21.5)	20.8 (17.4–22.9)	0.615	0.907	0.214	0.580	0.892	0.121
Surface expression of CCR7*											
Naïve ($CCR7^+$)	18.0 (14.5–23.2)	18.0 (13.9–22.0)	16.7 (14.9–23.7)	18.1 (14.7–21.1)	20.4 (17.2–22.2)	0.594	0.665	0.182	0.611	1.000	0.137
Differentiated ($CCR7^-$)	0.4 (0.3–0.9)	0.5 (0.3–0.9)	0.4 (0.3–0.6)	0.3 (0.2–0.5)	0.4 (0.3–0.7)	0.528	*0.003*	0.411	0.094	0.915	0.060
Surface expression of CCR3 and CCR6											
$CXCR5^+CD4^+$ Th1 ($CCR3^+CCR6^-$)	36.2 (30.7–42.4)	37.6 (30.9–44.0)	34.1 (30.3–37.8)	31.4 (28.8–36.8)	31.7 (27.3–35.5)	0.204	*0.003*	*0.012*	0.339	0.280	0.538
$CXCR5^+CD4^+$ Th17 ($CCR3^-CCR6^+$)	20.5 (16.6–26.7)	19.4 (15.8–24.7)	25.6 (20.4–28.6)	22.9 (18.9–27.3)	20.9 (17.6–29.1)	*0.036*	*0.024*	0.240	0.465	0.351	0.547
$CXCR5^+CD4^+$ $CCR3^+CCR6^+$	9.9 (7.0–12.8)	9.3 (6.8–12.7)	10.0 (7.7–14.0)	8.8 (6.5–10.7)	8.2 (5.8–10.1)	0.537	0.106	0.078	0.064	0.051	0.563
$CXCR5^+CD4^+$ $CCR3^-CCR6^-$	31.9 (26.8–38.1)	32.3 (26.2–38.4)	31.7 (27.2–33.2)	35.0 (31.2–41.9)	37.0 (31.5–43.3)	0.515	0.054	*0.026*	*0.019*	*0.009*	0.418
$CXCR5^+CD8^+$ T cells (within $CD8^+$ T cells)	2.9 (1.9–3.7)	2.9 (1.8–3.6)	2.6 (2.0–4.2)	2.8 (2.1–4.2)	3.4 (2.0–3.8)	0.949	0.534	0.360	0.650	0.716	0.755
Surface expression of CCR7											
Naïve ($CCR7^+$)	1.9 (1.2–2.9)	2.0 (1.2–3.0)	1.8 (1.2–2.8)	1.9 (1.4–3.2)	2.1 (1.5–3.2)	0.707	0.757	0.608	0.578	0.419	0.713
Differentiated ($CCR7^-$)	0.6 (0.4–1.0)	0.6 (0.3–0.9)	0.6 (0.5–1.5)	0.8 (0.5–1.1)	0.8 (0.3–1.4)	0.249	0.106	0.535	0.850	0.516	0.745
IL-21 production											
$CD4^+$											
$IL21^+$	12.5 (8.5–14.9)	13.3 (10.1–16.3)	8.5 (6.9–13.4)	9.6 (7.5–12.6)	9.7 (6.3–11.9)	*0.015*	0.000	*< 0.001*	0.707	1.000	0.664
$IL21^+IL17^+$	0.7 (0.6–0.9)	0.7 (0.6–0.9)	0.7 (0.4–1.2)	0.7 (0.4–1.0)	0.7 (0.3–1.1)	0.721	0.549	0.603	0.971	0.695	0.875
$CD8^+$											
$IL21^+$	4.3 (2.3–5.8)	4.6 (2.5–7.4)	3.5 (1.3–4.8)	3.1 (2.1–4.4)	2.8 (1.0–4.4)	0.071	*0.009*	*0.008*	0.787	0.537	0.317
$IL21^+IL17^+$	0.3 (0.2–0.5)	0.4 (0.2–0.5)	0.2 (0.1–0.4)	0.2 (0.2–0.5)	0.3 (0.2–0.8)	*0.033*	0.101	0.900	0.287	0.059	0.176
B cell subsets											
IgD/CD27 (within B cells)											
Naïve	66.6 (51.6–77.4)	70.3 (52.7–78.5)	62.7 (46.9–74.8)	67.3 (51.0–75.7)	53.0 (43.2–69.2)	0.281	0.429	*0.032*	0.546	0.282	0.066
Memory	29.5 (20.2–45.2)	27.8 (19.5–44.3)	33.4 (23.1–48.8)	30.8 (22.1–44.9)	44.5 (27.4–54.7)	0.212	0.292	*0.011*	0.670	0.199	*0.042*

Table 2 (continued)

	Sjögren's syndrome			Sicca	HC	Group's comparisons (*p* values)				
	Total SjS *n* = 53	SSA+SjS *n* = 38	SSA−SjS *n* = 15	*n* = 72	*n* = 24	SSA+ vs SSA−	SSA+ vs Sicca	SSA+ vs HC	SSA− vs Sicca	SSA− vs HC	Sicca vs HC
Unswitched memory	13.5 (8.7–21.6)	13.1 (8.4–20.6)	14.8 (10.2–25.5)	15.9 (11.0–24.9)	21.6 (14.6–32.2)	0.342	0.074	*0.004*	0.796	0.144	0.078
Switched memory	14.5 (10.0–22.5)	13.3 (9.8–21.6)	18.6 (12.2–24.1)	14.5 (10.2–21.6)	19.5 (13.7–26.5)	0.243	0.741	0.055	0.217	0.733	*0.049*
$CD24^{Hi}CD38^{Hi}$	6.3 (2.8–9.6)	6.3 (2.2–11.0)	4.2 (3.2–7.2)	4 (2.2–5.5)	3.2 (2.4–5.8)	0.518	*0.006*	0.058	0.146	0.194	0.819
$CD24^{Hi}CD27^{+}$	16.9 (10.1–27.6)	15.0 (9.0–24.7)	19.6 (12.7–38.4)	21.3 (14.0–34.1)	32.0 (21.7–42.5)	0.173	*0.014*	*< 0.001*	0.813	0.122	*0.031*
Plasmablasts $IgM^{-/+}CD38^{++}$	2.0 (0.9–3.5)	2.5 (1.2–4.5)	1.9 (0.7–2.7)	1.2 (0.7–1.9)	1.4 (1.0–1.9)	0.114	*0.001*	*0.032*	0.576	0.739	0.500

Mann-Whitney nonparametric *U* test was used for group's comparison. Results are presented as medians and interquartile range, median (IQR). Statistically significant results are indicated in italic

SjS, Sjögren's syndrome; *HC*, healthy controls; *IQR*, interquartile range

IL-21-secreting CD4 and CD8 T cells

We found increased percentages of $IL\text{-}21^{+}CD4^{+}$ T cells (13.3%) in SSA+SjS compared to SSA−SjS (8.5%, p = 0.015), *Sicca* (9.6%, $p < 0.001$), and HC (9.7%, p = 0.002), without differences in absolute counts. Similarly, $IL\text{-}21^{+}CD8^{+}$ T cells percentages were significantly increased in SSA+SjS (4.6%) compared to *Sicca* (3.1%, p = 0.009) and HC (2.8%, p = 0.008), and tendentially significant compared to SSA−SjS (3.12%, p = 0.071) (Fig. 3).

Contrary to SSA−SjS, SSA+SjS patients showed higher $IL\text{-}21^{+}CD8^{+}$ T cell counts compared to *Sicca* (p = 0.031). Furthermore, ESSDAI positively correlated with both $IL\text{-}21^{+}CD4^{+}$ (r = 0.456, p = 0.038) and $IL\text{-}21^{+}CD8^{+}$ (r = 0.451, p = 0.046) T cells in SSA+SjS patients with clinically active disease, but not in SSA−SjS (Fig. 4).

B cell subsets

The levels of total B cells were similar between groups, though increased percentages of B cells were observed in *Sicca* compared to SSA−SjS (p = 0.023), and decreased counts were present in SSA+SjS compared to HC (p = 0.033). We found no differences in the distinct IgD/CD27 B cell subsets between SSA+SjS and SSA−SjS. Interestingly, SSA+SjS patients showed increased naïve (p = 0.032) and decreased unswitched memory B cell percentages (p = 0.004) compared to HC. No differences were observed between SSA−SjS and the remaining groups, though these patients presented lower unswitched and switched memory B cell counts than HC ($p \leq 0.025$). *Sicca* patients also presented decreased percentages and absolute counts of total memory ($p \leq 0.042$) and switched memory ($p \leq 0.049$) B cells compared to HC.

When analyzing subsets related to regulatory B cells, memory $CD24^{Hi}CD27^{+}$ B cells showed lower counts and percentages in SSA+SjS patients than in *Sicca* ($p \leq 0.014$) and HC (< 0.001). $CD24^{Hi}CD27^{+}$ B cell percentages were similar in SSA−SjS and HC, although SSA−SjS presented decreased counts (p = 0.006). $CD24^{Hi}CD38^{Hi}$ B cell percentages were higher in SSA+SjS compared to *Sicca* (p = 0.006).

Concerning $IgM^{-/+}CD38^{Hi}$ plasmablasts, SSA+SjS showed increased percentages compared to *Sicca* (p = 0.001) and HC (p = 0.032).

Discussion

SjS patients display great clinical and biological heterogeneity [17].

We explored the clinical and immunological features of SSA+/SSA− patients focusing on the lymphocyte subpopulations distribution and its association with disease activity.

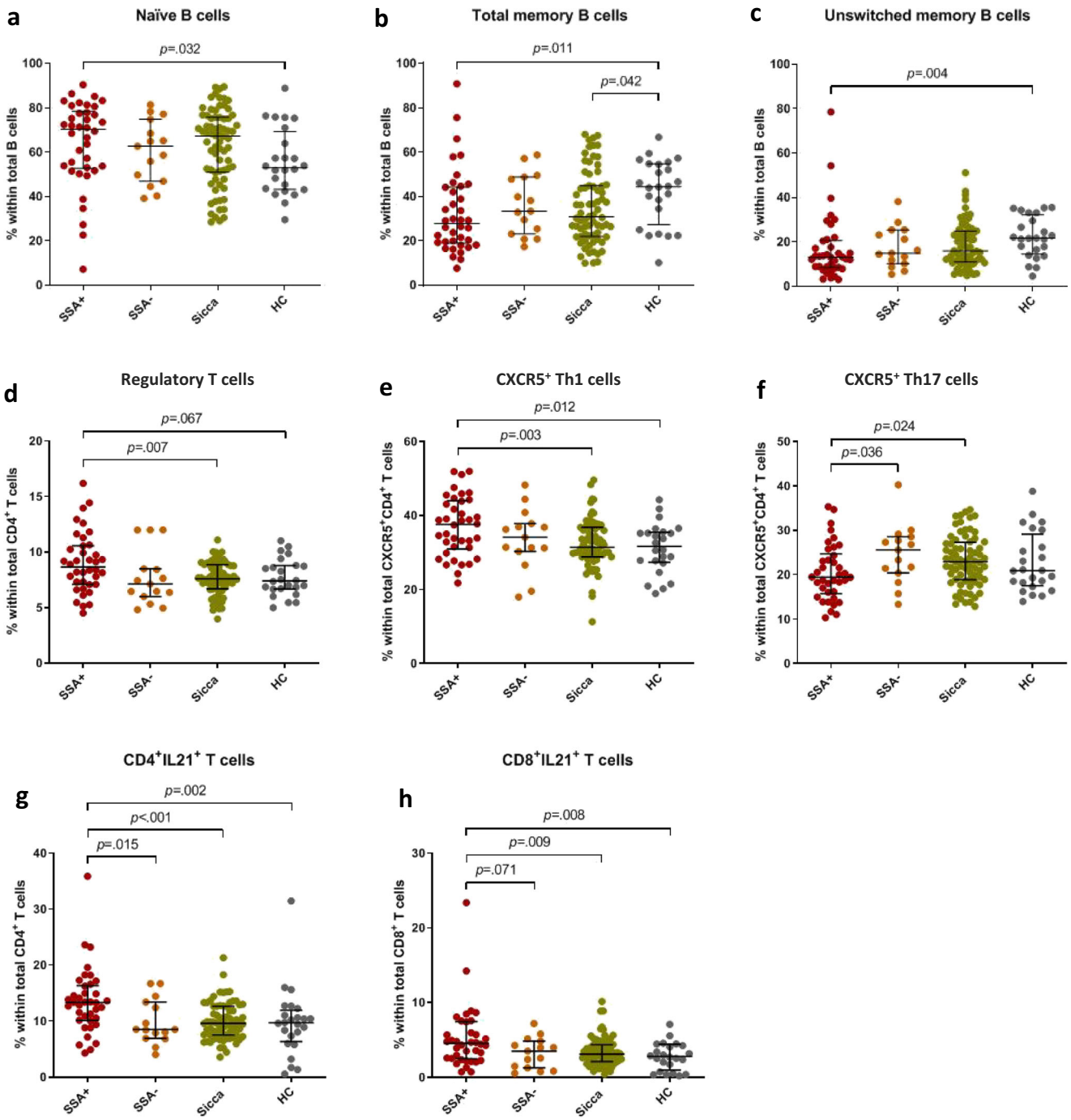

Fig. 3 Scatter dot plots with the distributions of T and B cell subsets' percentages. Scatter dot plots (median with interquartile ranges) with the distributions of T (d–h) and B (a–c) cell subsets percentages in all groups. Differences were tested using the Mann-Whitney *U* test

In our study, SSA+SjS patients presented higher proportions of IL-21-producing $CD4^+$ and $CD8^+$ T cells, a pronounced decrease in memory B cells, and increased naïve B cells, compared to HC. Moreover, SSA+SjS patients presented higher disease activity, and associations between ESSDAI and several lymphocyte subsets, including IL-21^+-producing $CD4^+$ and $CD8^+$ T cells, and Tregs. To our knowledge, this is the first study to approach $CXCR5^+$Tc cells in SjS patients according to anti-SSA positivity.

In SjS, the presence of anti-Ro/SSA and anti-La/SSB is a dominant feature, reflecting the hyperactivity of autoreactive B cells/plasma cells [18, 19]. Anti-SSA antibodies are associated with younger age of disease onset, worst exocrine gland function, and parotid gland enlargement [20], as well as

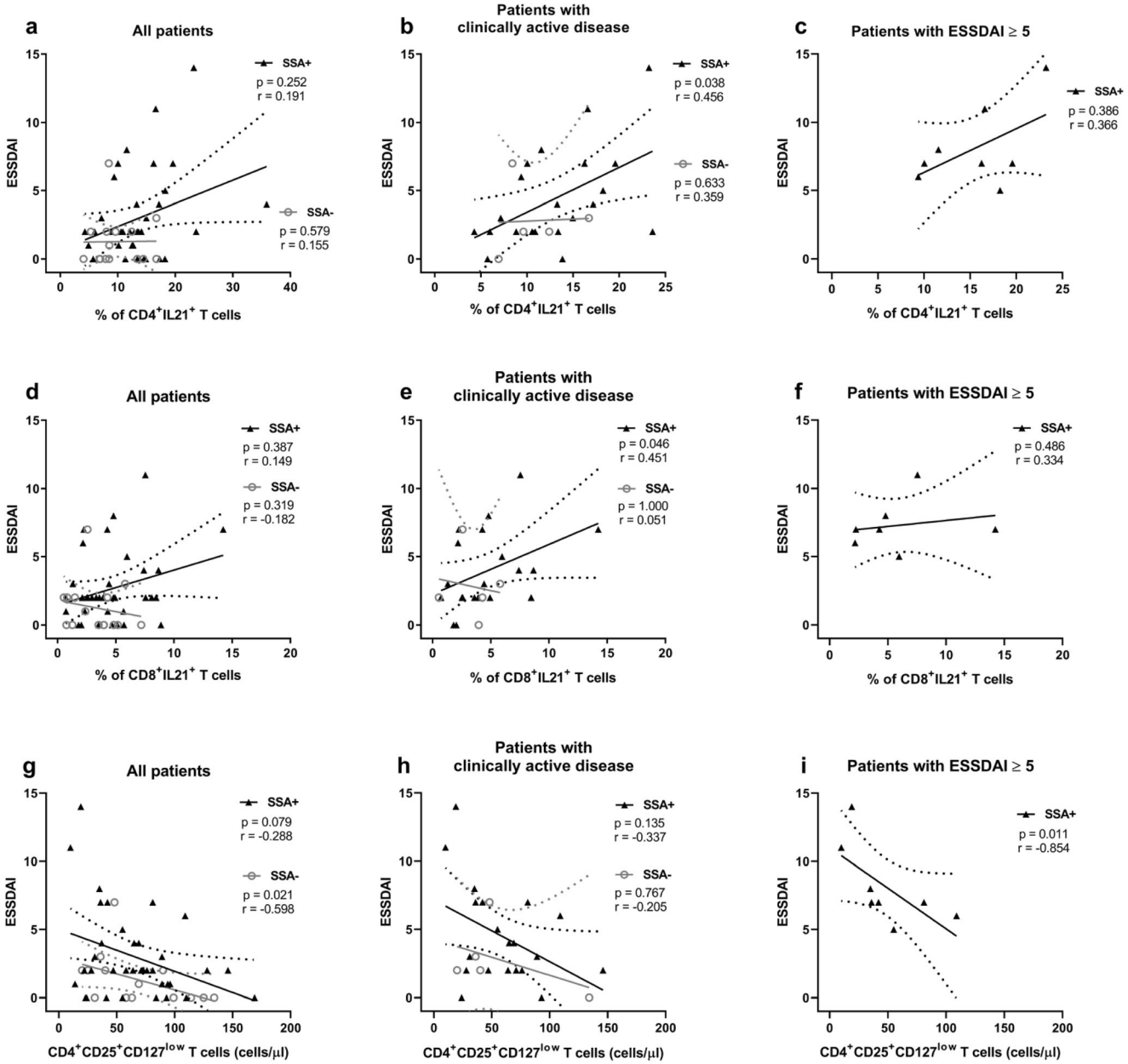

Fig. 4 Correlation of the ESSDAI with IL21-secreting $CD4^+$ and $CD8^+$ T cells, and $CD4^+CD25^+CD127^{low}$ T cells. ESSDAI score correlations with IL21-secreting $CD4^+$ and $CD8^+$ T cells, and $CD4^+CD25^+CD127^{low}$ T cells in all patients (a, d, and g), in patients with clinically active disease (b, e, and h), and in patients with ESSDAI ≥ 5 (c, f, and i). Correlations were performed in SSA+ and SSA− SjS subgroups. Spearman correlation coefficients, 95% confidence interval, and *p* values are indicated

several extra-glandular manifestations [13]. An increased risk of lymphoma is predicted by clinical adverse prognostic factors, along with the disease activity (ESSDAI scores) [21].

Our SSA+SjS patients had a younger age of onset and, conversely, a longer disease duration compared to SSA−SjS. ESSDAI was higher in SSA+SjS, but due to the small sample, clinical differences were not statistically significant, although SG swelling and Raynaud's phenomenon only occurred in SSA+SjS. RF and hypergammaglobulinemia were more prevalent in SSA+SjS. All these differences are in accordance with published data [13, 20].

In SjS patients, we found not only T cell lymphopenia but also decreased B cell counts, which also occurred to a lesser degree in *Sicca* patients.

Mandl [22] found significantly lower $CD4^+$ T cell counts and percentages in SjS, and lower absolute $CD4^+$ T cell counts in SSA+SjS, compared to *Sicca* patients. Another study showed that SSA+/SSB+SjS patients had decreased circulating $CD4^+$ T cells compared to SSA−/SSB−SjS and HC [23]. Also, $CD4^+$ T cell numbers were lower in both SjS groups compared to HC and in SSA+/SSB+SjS compared to SSA−/SSB−SjS [23]. Our findings are partially in line with these

studies, as both SSA+SjS and SSA−SjS groups had lower $CD4^+$ T cells compared to *Sicca* and HC groups. Interestingly, $CD4^+$ T cell counts were also lower in *Sicca* compared to HC.

Lymphopenia in SjS has been attributed to the migration of peripheral blood $CD4^+$ T cells to the exocrine glands [24] and therefore may traduce a more active disease profile. However, it has been recently proposed that decreased T cells in SjS represent a senescent phenotype due to earlier increased proliferation on naïve $CD4^+$ T cells contributing to their later exhaustion [25]. Epigenetic cell counting, a promising novel tool to quantify immune cells in the salivary glands, may contribute to a better understanding of cell distribution and immunopathology [26].

The higher percentages of $CD8^+$ T cells found in both SjS groups compared to *Sicca* and HC differ from Mandl et al. [22] study, which found no differences in $CD8^+$ T cells percentages while describing, as we did, lower $CD8^+$ T cell counts in both SSA+ and SSA−SjS [22].

Follicular T cells participate in T cell–dependent B cell responses in ectopic lymphoid structures. Typically, they express the follicular homing chemokine receptor CXCR5, which guides their migration into B cell follicles, and secrete the B cell helper cytokine IL-21 [27].

Increased Tfh frequencies have been described in the blood and SG of SjS (recently reviewed by Verstappen [5]), possibly related to extra-glandular manifestations [5, 28]. Our results showed no differences concerning $CXCR5^+CD4^+$ T cell frequencies between SjS (SSA+ and SSA−) and the other groups, while lower numbers were found in SSA+SjS compared to *Sicca* and HC. When addressing $CXCR5^+$Th1 and Th17 profiles, according to CXCR3 and CCR6 expression, we found higher frequencies of $CXCR5^+$Th1 and lower frequencies of $CXCR5^+$Th17 in SSA+SjS patients, especially when comparing SSA+SjS with SSA−SjS patients. Moreover, SSA+SjS had lower $CXCR5^+$Th17 counts compared to the other groups, and Th17 percentages were lower in SSA+SjS compared to SSA− and *Sicca*.

Despite some reports describe decreased circulating Tfh1 in SjS patients compared to HC, and higher or similar Tfh17 levels [29, 30], Aqrawy [31] observed a significant decrease of $CXCR5^+$Th17 frequencies in SjS. Our data are consistent with the later study, supporting possible retention of $CXCR5^+$Th17 cells in the SG at the site of inflammation, thus decreasing their circulating levels. The lower frequencies found in SSA+SjS patients might traduce a more intense SG accumulation in these patients, although a more specific phenotypic characterization of Tfh cells (PD1 marker) was not performed.

A positive correlation between ESSDAI and $CXCR5^+$Th1 percentages was observed in SSA+SjS patients, stronger in patients with moderate/high disease activity. By contrast, a negative correlation between ESSDAI and $CXCR5^+$Th17 percentages was present. Previous reports correlated Tfh17-like cells with disease activity and antibody production, suggesting also increased expression of effector cell markers such as PD1, CD40L, and IL-21 in these cells compared to Tfh1 cells [30]. Since the associations between ESSDAI score and $CXCR5^+$Th subsets only occurred in SSA+SjS patients, we admit that the immune mechanisms underlying T cell dynamics and disease manifestations may differ according to the presence of anti-SSA antibodies.

Follicular T cells are major sources of IL-21, an autocrine cytokine that regulates GC responses, B cell activation, and immunoglobulin production [32]. Thus, we characterized IL-21 production by $CD4^+$ and $CD8^+$ T cells to identify T cells with a follicular phenotype. SSA+SjS patients presented higher percentages of IL-21-secreting $CD4^+$ T cells compared to all other groups, and higher percentages of IL-21-secreting $CD8^+$ T cells compared to *Sicca* and HC. Our results suggest that, among SjS patients, those SSA+ may have a different biological and clinical profile, with more intense GC-like reactions. This is in line with a recent study by Pontarini [33], which showed that circulating $CXCR5^+ICOS^+PD\text{-}1^+$ Tfh subsets producing high levels of both IL-21 and IFN-γ were enriched in SjS patients with anti-Ro/SSA and anti-La/SSB autoantibodies, especially those with ectopic lymphoid structures, and positively correlated with SG focus score.

Follicular $CD8^+$ T cells have been identified in the synovial ectopic follicles of patients with rheumatoid arthritis [34], and recent data confirmed an important role of $CD8^+$ T cells in the development and progression of the SG lesions in SjS [35]. Our data, showing an increased follicular function in SSA+SjS patients, assessed by IL-21 production by $CD4^+$ and $CD8^+$ T cells, support an important role of both subsets for antibody production by the infiltrating B cells.

Noteworthy, we have also proven a strong positive correlation between ESSDAI and both $IL\text{-}21^+CD4^+$ and $IL\text{-}21^+CD8^+$ T cells in SSA+SjS patients with clinically active disease, a feature not found in SSA−SjS patients. This is in accordance with Mingueneau's study [24], where the frequencies of circulating HLA-DR-expressing activated $CD8^+$ and $CD4^+$ T cells correlated with the ESSDAI. Our observation that $IL\text{-}21^+CD8^+$ T cells presented the same association with disease activity as $IL\text{-}21^+CD4^+$ T cells suggest a role of these cells in B cell responses. Therefore, the increase in circulating follicular-related $CD8^+$ T cells in SSA+SjS probably indicates their role in the active clinical profile of these patients. Considering the fundamental role of IL-21 in GC dynamics, inducing B cell generation and differentiation into plasma cells, and that increased serum levels of IL-21 have been detected in patients with immune-mediated diseases, including SjS[36], our findings of increased percentages of IL-21-producing $CD4^+$ and $CD8^+$ T cells in SSA+SjS patients are not surprising.

For Treg characterization, we used the phenotype $CD4^+CD25^+CD127^{low}$ recommended by the Human Phenotyping Project [37], as these cells significantly express Foxp3 and correlate positively with the classic regulatory T cell $CD4^+CD25^+Foxp3^+$ [8].

Although both groups of SjS, as well as *Sicca* patients, had lower Treg counts compared to HC, SSA+SjS patients presented higher percentages compared to the other groups. Gottenberg [38] had previously described a higher proportion of functional $CD4^+CD25^{high}$ regulatory T cells in SjS, especially in patients with extra-glandular manifestations or anti-SSA/SSB antibodies, suggesting reactive feedback. Recently, higher levels of Tregs have also been reported by Maria [39] (in SjS patients with positive Interferon gene expression signature) and Verstappen [40]. However, Tregs are controversial in SjS [9]. Sudzius [23] did not find significant differences in circulating $CD4^+CD25^+Foxp3^+$ T cells in SjS patients with and without anti-SSA antibodies. Nevertheless, in their study, both patients and controls presented low levels of $CD4^+CD25^+Foxp3^+$ T cells (< 1.5% of total CD4 T cells) [41].

A negative correlation between the frequency of circulating $CD4^+CD25^+Foxp3^+$ cells and the proportion of Tregs infiltrating the SG was reported in SjS [42]. Moreover, patients with advanced pathologic lesions had higher circulating levels of $Foxp3^+$ cells and tissue infiltration by Tregs was reduced. Additionally, this study showed that $CD4^+CD25^+CD127^{low}$ T cell frequencies paralleled $Foxp3^+$ T cells in SjS and controls, supporting our choice for the $CD4^+CD25^+CD127^{low}$ phenotype.

Interestingly, in SSA+SjS with moderate/high disease activity, we found a strong negative correlation between ESSDAI and circulating Treg counts. Considering our data and the previous observations from Christodoulou [42], activity scores seem to proportionally increase to the level of infiltrating Tregs, relating to a decrease in circulating levels. Nevertheless, it cannot be excluded that the association between decreased Treg counts and ESSDAI may be simply a reflex of the lymphopenia in patients with more active disease. However, other authors failed to encounter associations between disease activity and circulating Tregs [9].

SSA+SjS patients presented several features of B cell activation, in accordance with our previous data [16], with higher proportions of naïve mature B cells and lower memory B cells compared to HC.

The increase in naïve mature B cells is widely described for SjS, and has been attributed to impairment of early B cell tolerance checkpoints [3]. Higher proportions of naïve B cells in SSA+SjS support a more severe immune dysregulation. The mobilization of self-reactive naïve B cells from the bone marrow to the periphery is increased in SjS [3], and recently Glauzy [43] found that among the expanded naïve B cells in SjS, most clones were polyreactive, pointing to their emergence from defective central and peripheral B cell tolerance checkpoints in SjS patients.

Also, increased migration of memory B cells to the affected SG occurs [44], accompanied by a shift in B cell differentiation towards plasma cells [45]. The consequent increased production of autoantibodies in the SG appears to strongly reflect the serologic humoral response [46]. Therefore, we admit that our SSA+SjS patients have a greater reduction in circulating memory B cells due to their migration to the SG, reinforced by the more frequent occurrence of SG enlargement and hypergammaglobulinemia, both of which are markers of B cell activation in SjS [47].

Additionally, $IgM^{-/+}CD38^{Hi}$ plasmablast percentages were increased in SSA+SjS compared to both *Sicca* and HC, whereas no differences were found comparing SSA−SjS. This is in line with the proposed identification of this population as plasma cell precursors, responsible for antibody production in SjS.

SSA−SjS also presented increased naïve and decreased memory B cell percentages and numbers compared to HC, although the small number of patients did not allow for further clarification.

The frequencies of new emigrant/transitional B cells expressing polyreactive B cell receptors were shown to be increased in SjS patients, a fact attributed to defective central B cell tolerance in SjS [43]. Considering $CD24^{Hi}CD38^{Hi}$ B cells as transitional cells, their significant increase in SSA+SjS may suggest a greater defect in central B cell tolerance allowing for their increased release into the circulation.

$CD24^{Hi}CD38^{Hi}$ B cells, as well as $CD24^{Hi}CD27^+$ B cells, are populations known to be enriched in regulatory cells [48]. In our study, decreased numbers and percentages of $CD24^{Hi}CD27^+$ B cells and increased $CD24^{Hi}CD38^{Hi}$ B cells occurred in SSA+SjS, whereas SSA−SjS only presented decreased numbers of $CD24^{Hi}CD27^+$ B cells. Our results on these populations are difficult to interpret as functional assays for IL-10 production were not performed. However, decreased $CD24^{hi}CD27^+$ B cells and a concomitantly reduced frequency of $IL10^+CD24^{hi}CD27^+$ B cells were reported in systemic lupus erythematosus (SLE) patients compared to HC [49]. The authors also reported decreased $CD24^{hi}CD38^{hi}$ B cell frequency in SLE patients, but no differences in $IL10^+CD24^{hi}CD38^{hi}$ B cells. Despite the changes found for $CD24^{Hi}CD38^{Hi}$ and $CD24^{Hi}CD27^+$ B cells in our study, the frequencies and function of IL-10-producing cells among those subsets remain undetermined.

We acknowledge limitations in our study, such as the inclusion of patients with a broad range of age and disease duration, variable disease activity and severity, and exclusive peripheral blood evaluation. Additionally, a significant number of patients in both groups were under glucocorticoid and/or hydroxychloroquine therapy, which could potentially influence lymphocyte subset distribution, although we consider

our population to be representative of the daily clinical practice. We also recognize the potential risk of bias due to multiple paired testing in our results. Finally, the use of different markers and phenotypic approaches can explain inconsistencies with the literature, and the absence of functional assays for regulatory T and B cells may overlook potential functional deficiencies in our study.

Our data support that SSA+SjS patients have more active disease, including markers of B cell activity such as hypergammaglobulinemia, RF, and SG enlargement. Overall, it seems that the underlying immune mechanisms differ according to the presence of anti-SSA antibodies, with a distinct lymphocyte subset profile observed in SSA+SjS patients. The more pronounced naïve/memory B cell imbalance in SSA+SjS patients reinforces the relevance of the expanded follicular T cell compartment and B cell activity. Furthermore, the association between ESSDAI and IL-21^{+}CD4^{+} and IL-21^{+}CD8^{+} follicular-related T cells suggests the possible involvement of these cells in the pathogenesis of the disease and their possible application for prognosis and monitoring of disease activity, particularly in SSA+SjS patients.

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Authors' contribution F Barcelos and C Martins conceived the original research idea, while all of the authors designed the study and created the study protocol. F Barcelos, J Vaz-Patto, and N Madeira recruited the patients and collected the data. J Cardigos and N Alves recruited the healthy controls and collected the data. C Martins analyzed the blood samples using flow cytometry. C Martins and M Ângelo-Dias performed the statistical analysis. JC Branco and L-M Borrego supervised all the work and the research protocol. All of the authors contributed to data analysis and interpretation. F Barcelos and N Madeira drafted the manuscript, and all of the authors revised it and contributed to it intellectually. All of the authors have approved the final version of the manuscript.

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Compliance with ethical standards

This study was approved by the Ethics committee of *Hospital CUF Descobertas,* 8/09/2014, Ethics committee of *Instituto Português de Reumatologia,* 3/07/2015 and NOVA Medical School Ethics (no 17/2016/CEFCM).

Disclosures None.

Consent to participate All patients have signed an informed consent to participate, according to the Declaration of Helsinki.

Consent to publication All authors have revised the final version of the manuscript and gave the consent to publication.

References

1. Ramos-Casals M, Brito-Zeron P, Siso-Almirall A, Bosch X (2012) Primary Sjogren syndrome. Bmj 344:e3821–e3821. https://doi.org/10.1136/bmj.e3821
2. Shiboski CH, Shiboski SC, Seror R, Criswell LA, Labetoulle M, Lietman TM, Rasmussen A, Scofield H, Vitali C, Bowman SJ, Mariette X, the International Sjögren's Syndrome Criteria Working Group, Heidenreich AM, Lanfranchi H, Vollenweider C, Schiødt M, Devauchelle V, Gottenberg JE, Saraux A, Pincemin M, Dörner T, Tzoufias A, Baldini C, Bombardieri S, de Vita S, Kitagawa K, Sumida T, Umehara H, Bootsma H, Kruize AA, Radstake TR, Vissink A, Jonsson R, Ramos-Casals M, Theander E, Challacombe S, Fisher B, Kirkham B, Larkin G, Ng F, Rauz S, Akpek E, Atkinson J, Baer AN, Carsons S, Carteron N, Daniels T, Fox B, Greenspan J, Illei G, Nelson D, Parke A, Pillemer S, Segal B, Sivils K, St.Clair EW, Stone D, Vivino F, Wu A (2017) 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 69:35–45. https://doi.org/10.1002/art.39859
3. Corsiero E, Sutcliffe N, Pitzalis C, Bombardieri M (2014) Accumulation of self-reactive naïve and memory B cell reveals sequential defects in B cell tolerance checkpoints in Sjögren's Syndrome. PLoS One 9:e114575. https://doi.org/10.1371/journal.pone.0114575
4. Salomonsson S, Jonsson MV, Skarstein K, Brokstad KA, Hjelmström P, Wahren-Herlenius M, Jonsson R (2003) Cellular basis of ectopic germinal center formation and autoantibody production in the target organ of patients with Sjögren's syndrome. Arthritis Rheum 48:3187–3201. https://doi.org/10.1002/art.11311
5. Verstappen GM, Kroese FGM, Bootsma H (2019) T cells in primary Sjögren's syndrome: targets for early intervention. Rheumatology 1–11 https://doi.org/10.1093/rheumatology/kez004
6. Zotos D, Coquet JM, Zhang Y, Light A, D'Costa K, Kallies A, Corcoran LM, Godfrey DI, Toellner KM, Smyth MJ, Nutt SL, Tarlinton DM (2010) IL-21 regulates germinal center B cell differentiation and proliferation through a B cell–intrinsic mechanism. J Exp Med 207:365–378
7. Quigley MF, Gonzalez VD, Granath A et al (2007) CXCR5+ CCR7- CD8 T cells are early effector memory cells that infiltrate tonsil B cell follicles. Eur J Immunol 37:3352–3362. https://doi.org/10.1002/eji.200636746
8. Yu N, Li XX, Song W, Li D, Yu D, Zeng X, Li M, Leng X, Li X (2012) CD4+CD25+CD127low/− T cells: a more specific Treg population in human peripheral blood. Inflammation 35:1773–1780. https://doi.org/10.1007/s10753-012-9496-8
9. Alunno A, Carubbi F, Bistoni O et al (2015) T regulatory and T helper 17 cells in primary Sjögren's syndrome: facts and perspectives. Mediators Inflamm. 2015:1–10
10. Bohnhorst JØ, Thoen JE, Natvig JB, Thompson KM (2001) Significantly depressed percentage of CD27+ (memory) B cells among peripheral blood B cells in patients with primary Sjogren's syndrome. Scand J Immunol 54:421–427. https://doi.org/10.1046/j.1365-3083.2001.00989.x
11. Achour A, Simon Q, Mohr A, Séité JF, Youinou P, Bendaoud B, Ghedira I, Pers JO, Jamin C (2017) Human regulatory B cells control the TFH cell response. J Allergy Clin Immunol 140:215–222. https://doi.org/10.1016/j.jaci.2016.09.042

12. Lin X, Wang X, Xiao F, Ma K, Liu L, Wang X, Xu D, Wang F, Shi X, Liu D, Zhao Y, Lu L (2019) IL-10-producing regulatory B cells restrain the T follicular helper cell response in primary Sjögren's syndrome. Cell Mol Immunol 16:921–931. https://doi.org/10.1038/s41423-019-0227-z
13. Ramos-Casals M, Solans R, Rosas J, Camps MT, Gil A, del Pino-Montes J, Calvo-Alen J, Jiménez-Alonso J, Micó ML, Beltrán J, Belenguer R, Pallarés L, GEMESS Study Group (2008) Primary Sjögren syndrome in Spain: clinical and immunologic expression in 1010 patients. Medicine (Baltimore) 87:210–219. https://doi.org/10.1097/MD.0b013e318181e6af
14. Seror R, Ravaud P, Bowman SJ, Baron G, Tzioufas A, Theander E, Gottenberg JE, Bootsma H, Mariette X, Vitali C, EULAR Sjögren's Task Force (2010) EULAR Sjogren's syndrome disease activity index: development of a consensus systemic disease activity index for primary Sjogren's syndrome. Ann Rheum Dis 69:1103–1109. https://doi.org/10.1136/ard.2009.110619
15. Seror R, Bootsma H, Saraux A, Bowman SJ, Theander E, Brun JG, Baron G, le Guern V, Devauchelle-Pensec V, Ramos-Casals M, Valim V, Dörner T, Tzioufas A, Gottenberg JE, Solans Laqué R, Mandl T, Hachulla E, Sivils KL, Ng WF, Fauchais AL, Bombardieri S, Priori R, Bartoloni E, Goeb V, Praprotnik S, Sumida T, Nishiyama S, Caporali R, Kruize AA, Vollenweider C, Ravaud P, Meiners P, Brito-Zerón P, Vitali C, Mariette X, EULAR Sjögren's Task Force (2016) Defining disease activity states and clinically meaningful improvement in primary Sjögren's syndrome with EULAR primary Sjögren's syndrome disease activity (ESSDAI) and patient-reported indexes (ESSPRI). Ann Rheum Dis 75:382–389. https://doi.org/10.1136/annrheumdis-2014-206008
16. Barcelos F, Martins C, Papoila A, Geraldes C, Cardigos J, Nunes G, Lopes T, Alves N, Vaz-Patto J, Branco J, Borrego LM (2018) Association between memory B-cells and clinical and immunological features of primary Sjögren's syndrome and Sicca patients. Rheumatol Int 38:1063–1073. https://doi.org/10.1007/s00296-018-4018-0
17. Quartuccio L, Baldini C, Bartoloni E, Priori R, Carubbi F, Corazza L, Alunno A, Colafrancesco S, Luciano N, Giacomelli R, Gerli R, Valesini G, Bombardieri S, de Vita S (2015) Anti-SSA/SSB-negative Sjögren's syndrome shows a lower prevalence of lymphoproliferative manifestations, and a lower risk of lymphoma evolution. Autoimmun Rev 14:1019–1022. https://doi.org/10.1016/j.autrev.2015.07.002
18. Tengnér P, Halse AK, Haga HJ, Jonsson R, Wahren-Herlenius M (1998) Detection of anti-Ro/SSA and anti-La/SSB autoantibody-producing cells in salivary glands from patients with Sjogren's syndrome. Arthritis Rheum 41:2238–2248. https://doi.org/10.1002/1529-0131(199812)41:12<2238::AID-ART20>3.3.CO;2-M
19. Stott DI, Hiepe F, Hummel M, Steinhauser G, Berek C (1998) Antigen-driven clonal proliferation of B cells within the target tissue of an autoimmune disease. The salivary glands of patients with Sjogren's syndrome. J Clin Invest 102:938–946. https://doi.org/10.1172/JCI3234
20. Tzioufas AG, Tatouli IP, Moutsopoulos HM (2012) Autoantibodies in Sjögren's syndrome: clinical presentation and regulatory mechanisms. Press Méd (Paris, Fr 1983) 41:e451–e460. https://doi.org/10.1016/j.lpm.2012.05.022
21. Nocturne G, Virone A, Ng WF, le Guern V, Hachulla E, Cornec D, Daien C, Vittecoq O, Bienvenu B, Marcelli C, Wendling D, Amoura Z, Dhote R, Lavigne C, Fior R, Gottenberg JE, Seror R, Mariette X (2016) Rheumatoid factor and disease activity are independent predictors of lymphoma in primary Sjögren's syndrome. Arthritis Rheumatol 68:977–985. https://doi.org/10.1002/art.39518
22. Mandl T, Bredberg A, Jacobsson LTH, Manthorpe R, Henriksson G (2004) CD4+ T-lymphocytopenia–a frequent finding in anti-SSA antibody seropositive patients with primary Sjögren's syndrome. J Rheumatol 31:726–728
23. Sudzius G, Mieliauskaite D, Siaurys A et al (2015) Distribution of peripheral lymphocyte populations in primary Sjögren's syndrome patients. J Immunol Res 2015:854706. https://doi.org/10.1155/2015/854706
24. Mingueneau M, Boudaoud S, Haskett S, Reynolds TL, Nocturne G, Norton E, Zhang X, Constant M, Park D, Wang W, Lazure T, le Pajolec C, Ergun A, Mariette X (2016) Cytometry by time-of-flight immunophenotyping identifies a blood Sjögren's signature correlating with disease activity and glandular inflammation. J Allergy Clin Immunol 137:1809–1821. https://doi.org/10.1016/j.jaci.2016.01.024
25. Fessler J, Fasching P, Raicht A, et al. (2020) Lymphopenia in primary Sjögren's syndrome is associated with premature aging of naïve CD4+ T cells. Rheumatology 1–10. https://doi.org/10.1093/rheumatology/keaa105
26. Blokland SLM, van Vliet-Moret FM, Hillen MR, Pandit A, Goldschmeding R, Kruize AA, Bouma G, van Maurik A, Olek S, Hoffmueller U, van Roon JAG, Radstake TRDJ (2020) Epigenetically quantified immune cells in salivary glands of Sjögren's syndrome patients: a novel tool that detects robust correlations of T follicular helper cells with immunopathology. Rheumatology (Oxford) 59:335–343. https://doi.org/10.1093/rheumatology/kez268
27. Yu D, Vinuesa CG (2010) The elusive identity of T follicular helper cells. Trends Immunol 31:377–383. https://doi.org/10.1016/j.it.2010.07.001
28. Szabo K, Papp G, Barath S, Gyimesi E, Szanto A, Zeher M (2013) Follicular helper T cells may play an important role in the severity of primary Sjögren's syndrome. Clin Immunol 147:95–104. https://doi.org/10.1016/j.clim.2013.02.024
29. Kurata I, Matsumoto I, Sumida T (2020) T follicular helper cell subsets: a potential key player in autoimmunity. Immunol. Med
30. Li X-y, Wu Z-b, Ding J et al (2012) Role of the frequency of blood CD4+CXCR5+CCR6+T cells in autoimmunity in patients with Sjögren's syndrome. Biochem Biophys Res Commun 422:238–244. https://doi.org/10.1016/j.bbrc.2012.04.133
31. Aqrawi LA, Ivanchenko M, Björk A, Ramírez Sepúlveda JI, Imgenberg-Kreuz J, Kvarnström M, Haselmayer P, Jensen JL, Nordmark G, Chemin K, Skarstein K, Wahren-Herlenius M (2018) Diminished CXCR5 expression in peripheral blood of patients with Sjögren's syndrome may relate to both genotype and salivary gland homing. Clin Exp Immunol 192:259–270. https://doi.org/10.1111/cei.13118
32. Long D, Chen Y, Wu H, Zhao M, Lu Q (2019) Clinical significance and immunobiology of IL-21 in autoimmunity. J. Autoimmun. 99:1–14
33. Pontarini E, Murray-Brown WJ, Croia C, Lucchesi D, Conway J, Rivellese F, Fossati-Jimack L, Astorri E, Prediletto E, Corsiero E, Romana Delvecchio F, Coleby R, Gelbhardt E, Bono A, Baldini C, Puxeddu I, Ruscitti P, Giacomelli R, Barone F, Fisher B, Bowman SJ, Colafrancesco S, Priori R, Sutcliffe N, Challacombe S, Carlesso G, Tappuni A, Pitzalis C, Bombardieri M (2020) 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 79:1588–1599. https://doi.org/10.1136/annrheumdis-2020-217646
34. Kang YM, Zhang X, Wagner UG, Yang H, Beckenbaugh RD, Kurtin PJ, Goronzy ÏJ, Weyand CM (2002) CD8T cells are required for the formation of ectopic germinal centers in rheumatoid synovitis. J Exp Med 195:1325–1336. https://doi.org/10.1084/jem.20011565
35. Gao CY, Yao Y, Li L, Yang SH, Chu H, Tsuneyama K, Li XM, Gershwin ME, Lian ZX (2019) Tissue-resident memory CD8+ T cells acting as mediators of salivary gland damage in a murine

model of Sjögren's syndrome. Arthritis Rheumatol 71:121–132. https://doi.org/10.1002/art.40676
36. Kang KY, Kim HO, Kwok SK, et al. (2011) Impact of interleukin-21 in the pathogenesis of primary Sjögren's syndrome: increased serum levels of interleukin-21 and its expression in the labial salivary glands. Arthritis Res Ther 13. https://doi.org/10.1186/ar3504
37. Finak G, Langweiler M, Jaimes M, Malek M, Taghiyar J, Korin Y, Raddassi K, Devine L, Obermoser G, Pekalski ML, Pontikos N, Diaz A, Heck S, Villanova F, Terrazzini N, Kern F, Qian Y, Stanton R, Wang K, Brandes A, Ramey J, Aghaeepour N, Mosmann T, Scheuermann RH, Reed E, Palucka K, Pascual V, Blomberg BB, Nestle F, Nussenblatt RB, Brinkman RR, Gottardo R, Maecker H, McCoy JP (2016) Standardizing flow cytometry immunophenotyping analysis from the Human ImmunoPhenotyping Consortium. Sci Rep 6. https://doi.org/10.1038/srep20686
38. Gottenberg J-E, Lavie F, Abbed K et al (2005) CD4 CD25high regulatory T cells are not impaired in patients with primary Sjögren's syndrome. J Autoimmun 24:235–242. https://doi.org/10.1016/j.jaut.2005.01.015
39. Maria NI, van Helden-Meeuwsen CG, Brkic Z, Paulissen SMJ, Steenwijk EC, Dalm VA, van Daele PL, Martin van Hagen P, Kroese FGM, van Roon JAG, Harkin A, Dik WA, Drexhage HA, Lubberts E, Versnel MA (2016) Association of increased Treg cell levels with elevated indoleamine 2,3-dioxygenase activity and an imbalanced kynurenine pathway in interferon-positive primary Sjögren's syndrome. Arthritis Rheumatol 68:1688–1699. https://doi.org/10.1002/art.39629
40. Verstappen GM, Meiners PM, Corneth OBJ, Visser A, Arends S, Abdulahad WH, Hendriks RW, Vissink A, Kroese FGM, Bootsma H (2017) Attenuation of follicular helper T cell–dependent B cell hyperactivity by abatacept treatment in primary Sjögren's syndrome. Arthritis Rheumatol 69:1850–1861. https://doi.org/10.1002/art.40165
41. Yi JS, Rosa-Bray M, Staats J, Zakroysky P, Chan C, Russo MA, Dumbauld C, White S, Gierman T, Weinhold KJ, Guptill JT (2019) Establishment of normative ranges of the healthy human immune system with comprehensive polychromatic flow cytometry profiling. PLoS One 14:e0225512. https://doi.org/10.1371/journal.pone.0225512
42. Christodoulou MI, Kapsogeorgou EK, Moutsopoulos NM, Moutsopoulos HM (2008) Foxp3+ T-regulatory cells in Sjögren's syndrome. Am J Pathol 173:1389–1396. https://doi.org/10.2353/ajpath.2008.080246
43. Glauzy S, Sng J, Bannock JM, Gottenberg JE, Korganow AS, Cacoub P, Saadoun D, Meffre E (2017) Defective early B cell tolerance checkpoints in Sjögren's syndrome patients. Arthritis Rheumatol 69:2203–2208. https://doi.org/10.1002/art.40215
44. Hansen A, Odendahl M, Reiter K, Jacobi AM, Feist E, Scholze J, Burmester GR, Lipsky PE, Dörner T (2002) Diminished peripheral blood memory B cells and accumulation of memory B cells in the salivary glands of patients with Sjögren's syndrome. Arthritis Rheum 46:2160–2171. https://doi.org/10.1002/art.10445
45. Szyszko EA, Brokstad KA, Øijordsbakken G et al (2011) Salivary glands of primary Sjögren's syndrome patients express factors vital for plasma cell survival. Arthritis Res Ther 13:R2. https://doi.org/10.1186/ar3220
46. Maier-Moore JS, Koelsch KA, Smith K, Lessard CJ, Radfar L, Lewis D, Kurien BT, Wolska N, Deshmukh U, Rasmussen A, Sivils KL, James JA, Farris AD, Scofield RH (2014) Antibody-secreting cell specificity in labial salivary glands reflects the clinical presentation and serology in patients with sjögren's syndrome. Arthritis Rheumatol 66:3445–3456. https://doi.org/10.1002/art.38872
47. Kroese FGM, Abdulahad WH, Haacke E, Bos NA, Vissink A, Bootsma H (2014) B-cell hyperactivity in primary Sjögren's syndrome. Expert Rev Clin Immunol 10:483–499. https://doi.org/10.1586/1744666X.2014.891439
48. Mauri C, Menon M (2017) Human regulatory B cells in health and disease: therapeutic potential. J. Clin. Invest. 127:772–779
49. Jin L, Weiqian C, Lihuan Y (2013) Peripheral CD24hi CD27+ CD19+ B cells subset as a potential biomarker in naive systemic lupus erythematosus. Int J Rheum Dis 16:698–708. https://doi.org/10.1111/1756-185X.12229