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Association between memory B-cells and clinical and immunological features of primary Sjögren's syndrome and Sicca patients

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Abstract

B-cells play a pivotal role in primary Sjögren's syndrome (pSS) pathogenesis. We aim to (1) evaluate the distribution of B-lymphocyte subpopulations in pSS and Sicca patients, (2) establish cut-off points that discriminate pSS from controls, (3) evaluate the association between memory B-cells and phenotypic features in pSS. We included 57 pSS patients, 68 Sicca and 24 healthy controls. Circulating B-cells were characterized by flow cytometry as naïve and memory subsets and classified from Bm1 to Bm5. Compared to controls, pSS patients had lower percentages (29.5 vs 44.4%) and absolute numbers (47 vs 106 cells/µl) of memory B-cells. Through ROC curves, a cut-off of ≤ 58 total memory B-cells/µl yielded a specificity of 0.88 and a sensitivity of 0.60 for pSS, and was met by 59.6% of pSS patients, 38.8% of Sicca and 12.5% of controls. A cut-off of < 23.5 Switched-memory B-cells/µl yielded a specificity of 0.88 and a sensitivity of 0.54 and was met by 54.4% of pSS patients, 37.3% of Sicca and 12.5% of controls. In pSS, lower total memory B-cells count was associated with longer disease duration (14.3 vs 8.1 years, $p = 0.006$) and more active disease profile, as evaluated by the European League Against Rheumatism (EULAR) Sjögren's Syndrome Disease Activity Index (ESSDAI) (3.1 vs 1.4, $p = 0.043$). Decreased numbers of memory B-cells clearly discriminated pSS from controls and can also have prognostic value. It remains to be clarified whether Sicca patients with decreased memory B-cells represent pSS and if B-cell profiling could help in the diagnosis of pSS.

Keywords Sjögren's syndrome · Flow cytometry · Memory B cells · Diagnosis · Autoimmunity

Introduction

Primary Sjögren's syndrome (pSS) is characterized by lymphocytic infiltration and damage of the exocrine glands, resulting in glandular dysfunction with xerostomia and xerophthalmia [1]. Systemic manifestations include arthritis, vasculitis, lung, neurological and renal involvement [2]. The main elements for pSS diagnosis are the presence of anti-SSA antibodies [3] and focal lymphocytic infiltrates in the minor salivary gland (MSG) biopsy [4, 5]. These have been included as mandatory items used in the classification criteria [6–8], from which the American-European Consensus Group (AECG) [6] have been the most used in the clinical practice for the last 15 years. Recently, a new set of criteria was proposed by the ACR/EULAR joint initiative [8], based on a weighted sum of objective items, which could potentially be adapted, if new diagnostic tests arise.

Clinical diagnosis remains difficult, especially in the early stages of the disease [9], since many patients with pSS may not meet the immunological criteria. Although anti-SSA antibodies can be detected before the recognition of clinical disease [10], they are negative in one-third of patients [11]. The histological criterion (focus score ≥ 1) has also variable sensitivity [4] and seems to be associated with the occurrence of anti-SSA, rheumatoid factor (RF) and antinuclear antibodies (ANA) [12]. Therefore, it is of great interest to identify and validate other instruments to support the diagnosis, especially in patients that although not fulfilling the classification criteria, may have the disease, as judged by the experts. Examples of potential new instruments are the study of lymphocytic subpopulations by flow cytometry [13, 14],

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the identification of new auto-antibodies [15] and salivary gland sonography [16].

The immunopathogenesis of pSS is complex and involves the innate immune system and both arms of the adaptive immune system, cellular and humoral [17]. B-cell disturbances are the hallmark of pSS and play a pivotal role in the disease pathogenesis and clinical evolution [18–20]. The hyperactivity of B-lymphocytes in pSS is recognized by hypergammaglobulinemia, cryoglobulinemia, cytokine and antibody production [21], and results in an increased risk of lymphoma [22].

B-lymphocytes leave the bone marrow as transitional B-cell and proceed to the secondary lymphoid organs, where their maturation continues under modulation of T-lymphocytes [23]. B cells in different stages of differentiation may be identified in peripheral blood based on their expression of distinct surface markers patterns. There is a great interest in identifying distinct B-cell subset profiles that may have a potential role for the diagnosis of autoimmune diseases [14]. In pSS, the distribution of peripheral B-cell subpopulations is altered, with an increase of the naive subset and the decrease of circulating memory cells [24–26]. A decreased frequency of memory cells has also been identified in patients with Sicca syndrome without criteria for pSS [25].

CD27 is a marker of somatically mutated B cells and memory B-cells. The expression of CD27, IgD and IgM allows the identification of naïve B-cells ($CD27^-IgM^+IgD^+$), unswitched ($CD27^+IgM^+IgD^+$) and switched ($CD27^+IgM^-IgD^-$) memory B-cells in the peripheral blood [27].

The Bm1–Bm5 classification of the $CD19^+$ B-cell compartment has also been used in the context of autoimmune diseases. According to the expression of CD38 and IgD, it allows the identification of several development stages of B cells which may leave the germinative centres and be detected in circulation [28], classified from Bm1 to Bm5 [29]. This classification partially overlaps with the IgD/CD27 classification. Bm1 includes transitional and naïve B-cells (IgD^+CD38^-), which become Bm2 (IgD^+CD38^+) upon activation and progress to germinal centre founder (IgD^+CD38^{++}). Bm3 centroblasts and Bm4 centrocytes (IgD^-CD38^+) are seldom identified in circulation and will differentiate inside the germinal centre into either plasma cells, or early and late memory cells (eBm5: IgD^-CD38^+ and Bm5: IgD^-CD38^-, respectively).

In pSS, specific changes in the distribution of these populations have been described, namely the increase of the Bm2 and Bm2′ subsets with a decrease of the eBm5 and Bm5 subpopulations [24]. In recent papers these changes have been suggested to be useful as diagnostic tools [30, 31].

Our study aims to evaluate the distribution of B-lymphocyte subpopulations in patients with pSS and Sicca syndrome through flow cytometry and to establish cut-off points for pSS classification in relation to healthy controls. Moreover, we aim to evaluate the relation between lymphocyte subpopulations and phenotypic features in pSS patients.

Materials and methods

Population

We have included adult patients followed at the Rheumatology department of *Instituto Português de Reumatologia* and *Hospital Cuf Descobertas*, with confirmed or suspected pSS. We have consecutively recruited patients, which were classified as pSS if they fulfilled the AECG classification criteria [6], or as non-Sjögren Sicca syndrome (designated as "Sicca group") if they did not fulfilled pSS criteria. The exclusion criteria from the AECG criteria were applied to all patients, and additional exclusion factors were considered for both groups: IgG_4-related disease, history of other corneal diseases and refractive surgery. Recruitment and evaluation has been performed between September 2014 and March 2017. The control group consisted of healthy women without Sicca symptoms, selected from the Ophthalmology outpatient clinic.

Disease activity in pSS patients was determined with the European League Against Rheumatism (EULAR) Sjögren's Syndrome Disease Activity Index (ESSDAI) [32]. pSS patients were stratified according to the disease activity in two subgroups: low disease activity (ESSDAI < 5) and moderate or high disease activity (ESSDAI ≥ 5) [33].

Informed consent has been obtained from all patients and controls.

This study was approved by the Ethics committee of *Hospital Cuf Descobertas*, Ethics committee of *Instituto Português de Reumatologia* and NOVA Medical School Ethics (no. 17/2016/CEFCM).

Flow cytometry measurements

A total of 6 ml of whole peripheral blood was collected into EDTA containing tubes. All samples were processed and analyzed within 24 h after collection.

To characterize B-lymphocyte subpopulations, a pre-validated panel of membrane markers was used, with the following monoclonal antibodies: CD19, CD24, CD27, CD38, Anti-IgD and Anti-IgM.

The acquisition of samples was performed using a 4-color BD FACS Calibur™ cytometer (BD Biosciences). Cell Quest Pro™ software (BD Biosciences) was used for both acquisition and analysis of samples.

In each tube, at least 5×10^3 $CD19^+$ events (B-lymphocytes) were acquired.

Each studied B-cell subset was evaluated in percentages and absolute counts. For the CD27/IgD classification (Fig. 1), naive, memory, unswitched memory and switched memory B-cells were evaluated (Fig. 1). As for the Bm1-5 classification, six other subsets were considered: Bm1, Bm2, Bm2′, Bm3 + Bm4, eBm5 and Bm5 B-cells. For absolute counts, a single-platform strategy was used, with BD Trucount tubes™.

Complete gating strategies are described in supplementary data and illustrated in Supplementary Fig. 1.

Statistical analysis

An exploratory analysis was carried out for all variables. Quantitative variables were described with mean and standard deviation (SD) or median and inter-quartile range (IQR: 25th percentile–75th percentile), as appropriate. Categorical data were presented as frequencies and percentages. To compare the distribution of the B-cell subsets between pSS, Sicca and control groups, Kruskal–Wallis test was applied, and whenever differences between at least two of these groups were identified, multiple comparisons were used to overcome the multiple testing problem. The Receiver Operating Characteristic (ROC) curves were used to establish cut-off points in the B-cells subset levels and to estimate corresponding sensitivity and specificity. Data analysis was performed using R (R: A Language and Environment for Statistical Computing, R Core Team, R Foundation for Statistical Computing, Vienna, Austria, year = 2017, http://www.R-project.org).

Results

Patients' characteristics

Fifty-seven patients were included in the pSS group, 68 in the Sicca group and 24 in the control group.

pSS mean age was 58.3 years with standard deviation (SD) of 11.9 years, and Sicca patients' mean age and SD were 60.5 and 10.7 years, respectively. In the control group, the mean age was 51.1 years with SD of 6.6 years. Average disease duration in pSS patients was 11.8 years with SD of 7.8 years, and in Sicca patients was 9.7 and 5.0 years, respectively. The clinical and immunological characteristics of both patient groups are presented in Table 1.

B-cell subsets according to IgD and CD27 classification

pSS and Sicca patients had lower absolute number of lymphocytes in comparison to controls ($p = 0.001$ and $p = 0.053$, respectively), as well as lower B-cell numbers, with

**Table 1** pSS and Sicca patients' characteristics

	pSS ($N = 57$)	Sicca ($N = 68$)
Ocular symptoms, n (%)	54 (94.7)	66 (95.6)
Oral symptoms, n (%)	55 (96.5)	65 (95.6)
Ocular signs, n (%)	34/56 (62.5)	35/66 (53.8)
Oral signs, n (%)	34/50 (70.8)	42/62 (67.7)
Parotid enlargement, n (%)	8 (14.0)	1 (1.5)
Extraglandular disease (ever), n (%)	23 (40.4)	24 (35.3)
Joint symptoms, n (%)	24 (42.1)	31 (47.7)
Skin involvement, n (%)	18 (31.6)	16 (24.6)
Other Extraglandular involvement, n (%)	5 (8.8)	0 (0)
Raynaud's phenomenon, n (%)	8 (14.0)	17 (26.2)
Focus score ≥ 1, n (%)	43/56 (79.6)	0 (0)
SSA, n (%)	38 (66.7)	1 (1.5)
SSB, n (%)	18/50 (36.0)	1/61 (1.6)
ANA $\geq 1/320$, n (%)	45 (78.9)	37 (54.4)
ANA $\geq 1/640$, n (%)	32 (56.1)	16 (23.9)
Rheumatoid factor, n (%)	24/50 (48.0)	18/66 (27.3)
Gammaglobulin ≥ 1.6 g/dl, n (%)	14 (24.6)	3/66 (4.5)
Therapy (any), n (%)	32 (56.1)	30 (46.2)
Glucocorticoids, n (%)	19 (33.3)	18 (26.5)
Hidroxichloroquine, n (%)	20 (35.1)	20 (29.4)
Imunossupressants, n (%)	10 (17.5)	8 (11.8)

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. Joint symptoms include arthritis and joint pain of inflammatory origin, but only cases that would score in the articular domain of ESSDAI were considered as extra-glandular disease. Likewise, in some patients skin involvement (which not included xerosis) was not considered as extra-glandular disease if it would not score in the cutaneous domain of ESSDAI

pSS, primary Sjögren's syndrome; SF, salivary flow; SSA/SSB, Sjögren's syndrome A/B antibody; ANA, antinuclear antibody; RF, rheumatoid factor; ESSDAI, European Sjögren's syndrome disease activity index

statistical significance when comparing pSS with controls ($p = 0.031$). There were no significant differences between both patient groups regarding total lymphocyte and B-cell numbers ($p = 0.490$ and $p = 0.165$, respectively) (Table 2). Regarding naïve B-cells, there were no significant differences between patient groups and controls (Table 2; Supplementary Table 1). Significant differences were found between pSS and controls in absolute counts of all memory populations: total memory (TMem) (CD19$^+$CD27$^+$), switched memory (SwM) (CD19$^+$IgD$^-$CD27$^+$) and unswitched memory (UnSwM) ($p < 0.001$ for all) (Table 2). Comparing pSS patients with controls, we found a weak evidence of lower percentages of TMem B-cells ($p = 0.078$) in patients, and more significant differences in the UnSwM subset ($p = 0.043$) (Supplementary Table 1). Percentages

Fig. 1 a Examples of typical blood memory B-cell subset profiling by flow cytometry in pSS patients, Sicca patients and controls. annotations of the figure: analyses are gated on IgD and CD27. **b** Box-plots with the distribution of absolute numbers and percentages of total Memory B-cells (b1 and b2, respectively) and of Switched memory B-cells (b3 and b4, respectively). pSS, primary Sjögren's syndrome
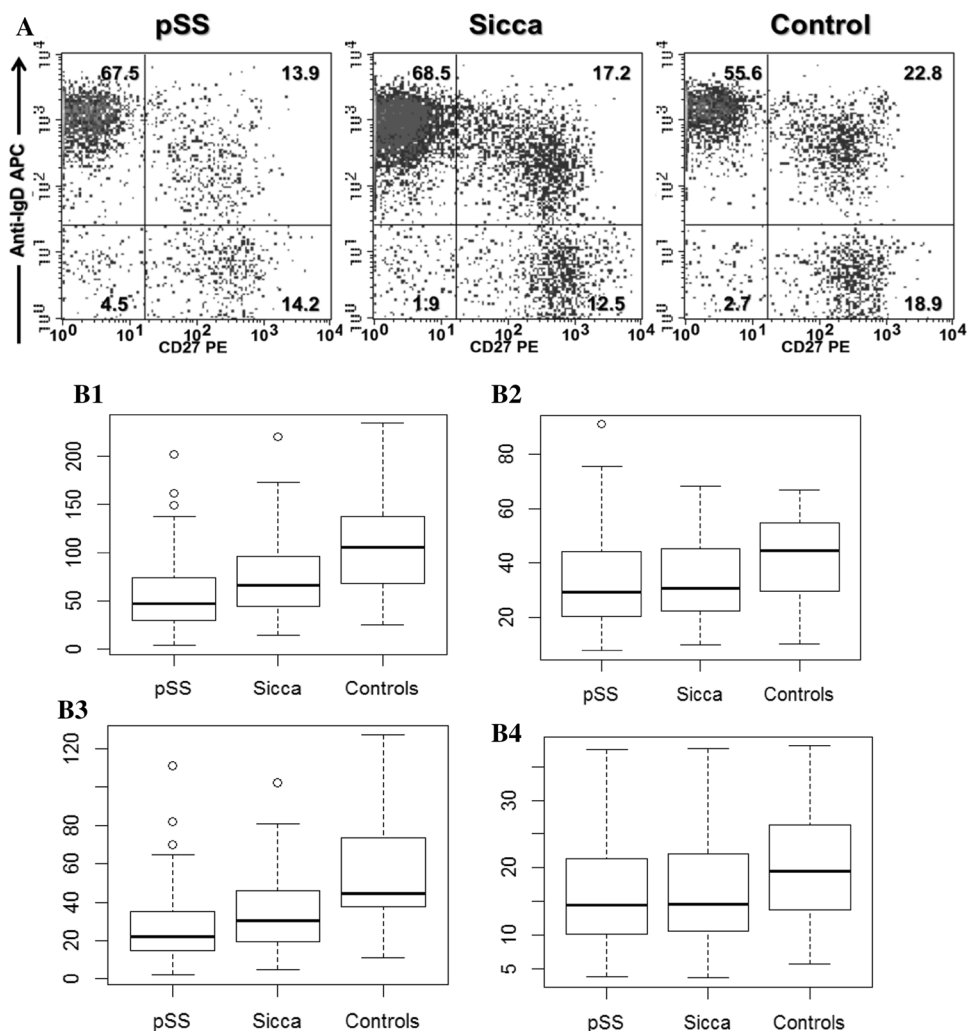
of memory B-cells in Sicca patients were similar to pSS patients (30.8 and 29.5%, respectively), and lower than controls (30.8 and 44.4%, respectively), although the difference was not statistical significant ($p = 0.300$) (Supplementary Table 1). Absolute memory B-cells numbers in Sicca were intermediate between those of pSS (66 and 47 cells/ μl, respectively, $p = 0.103$) and controls (66 and 106 cells/ μl, respectively, $p = 0.071$) (Fig. 1; Table 2), being more significant when considering the memory subsets SwM cells ($p = 0.053$ when comparing with controls) and UnSwM cells ($p = 0.058$ when comparing with pSS).

Bm1–Bm5 classification of mature b cells

In pSS patients, the percentage of Bm1 cells was lower than in controls and Sicca ($p = 0.067$ and $p = 0.064$, respectively) (Supplementary Table 1). The difference was even more significant for absolute counts (Table 2) ($p < 0.001$ versus controls, and $p = 0.002$ versus Sicca).

Regarding Bm2 and Bm2′ cells, no significant differences were found between the three groups, although in the pSS group the Bm2 count was lower than in the other two groups. The percentages of eBm5 and Bm5 cells did not differ between groups, but patients with pSS presented significantly lower numbers compared to controls ($p < 0.001$ for eBm5 and $p = 0.002$ for Bm5). Sicca patients presented values between those of pSS and controls, without statistical significance. Even though patients with pSS presented higher Bm2 + Bm2′/eBm5 + Bm5 ratios than controls, the difference was not statistically significant ($p = 0.613$).

Cut-off points for optimal diagnosis and best specificity

pSS patients and controls were compared and ROC curves were used to identify cut-off points with optimal sensitivity and specificity concerning naive and memory populations, as well as Bm1–Bm5 subsets. In most cases, the absolute

Table 2 Comparison of B-cell subsets absolute counts in pSS, Sicca syndrome and healthy controls

B-cell types	pSS	Sicca syndrome	Controls	Groups' comparisons (p value*)		
	n = 57	n = 68	n = 24	pSS vs controls	Sicca syndrome vs controls	pSS vs Sicca syndrome
Cells/μl, median (25th–75th percentile)						
Lymphocytes	1615 (1143–2312)	2005 (1509–2297)	2228 (1998–2287)	0.001	0.053	0.490
B cells	177 (109–261)	229 (143–308)	252 (173–354)	0.031	1	0.165
IgD/CD27						
Naive	108 (61–186)	138 (76–208)	137 (89–223)	0.647	1	0.478
Total memory	47 (29–74)	66 (44–96)	106 (71–134)	<0.001	0.071	0.103
Unswitched	22 (12–37)	35 (21–53)	57 (35–80)	<0.001	0.190	0.058
Switched	22 (15–35)	30 (20–46)	45 (38–71)	<0.001	0.053	0.182
Double-negative	3 (2–8)	4 (2–9)	4 (2–8)	1	1	1
Bm1–Bm5						
Bm1	18 (10–27)	28 (18–43)	38 (22–61)	<0.001	1	0.002
Bm2	101 (57–162)	138 (76–186)	130 (104–199)	0.245	1	0.389
Bm2′	11 (6–29)	11 (6–19)	12 (7–29)	1	1	1
Bm3 + Bm4	3 (1–5)	3 (2–4)	3 (2–4)	1	1	1
eBm5	15 (9–23)	18 (11–29)	27 (20–38)	<0.001	0.062	0.267
Bm5	11 (8–19)	16 (10–22)	24 (15–34)	0.002	0.143	0.331
Bm2 + Bm2′/Bm5 + eBm5	3.9 (2.2–6.9)	4.1 (2.1–5.9)	2.9 (1.9–4.1)	0.613	0.726	1

pSS, primary Sjögren's syndrome

*Obtained by Kruskal–Wallis test multiple comparisons

Table 3 Levels of B-cell subsets (absolute values) for optimal and high specificities for the classification of pSS

B-cell subsets or combinations of subsets	AUC (95% CI)	Optimal[a]			Specificity = 0.88	
		Cut-off point	Sensitivity	Specificity	Cut-off point	Sensitivity
IgD/CD27						
Naive	0.61 (0.48, 0.74)	107.00	0.49	0.71	63.00	0.28
Memory total	0.78 (0.67, 0.89)	72.00	0.74	0.75	58.00	0.60
Unswitched	0.76 (0.65, 0.88)	36.50	0.74	0.75	17.50	0.37
Switched	0.78 (0.67, 0,89)	35.50	0.75	0.79	23.50	0.54
Ratio naive/memory T	0.67 (0.55, 0.80)	1.52	0.65	0.71	3.38	0.33
Ratio naive/switched M	0.63 (0.50, 0.76)	3.17	0.63	0.62	8.00	0.23
Double-negative	0.52 (0.39, 0.66)	3.50	0.54	0.50	0.50	0.09
Bm1–Bm5						
Bm1	0.76 (0.63, 0.88)	27.50	0.77	0.71	13.63	0.42
Bm2	0.64 (0.52, 0.77)	104.50	0.53	0.75	77.50	0.39
Bm2′	0.53 (0.40, 0.66)	19.50	0.65	0.46	4.50	0.19
eBm5	0.77 (0.66, 0.89)	24.50	0.79	0.71	14.50	0.49
Bm5	0.74 (0.62, 0.86)	12.99	0.61	0.83	10.00	0.42
Bm2 + Bm2′	0.63 (0.50, 0.76)	132.50	0.60	0.62	82.50	0.37
eBm5 + Bm5	0.77 (0.65, 0.88)	38.00	0.72	0.75	23.33	0.47
Bm2 + Bm2′/Bm5 + eBm5	0.62 (0.49, 0.75)	3.83	0.53	0.75	6.77	0.28

AUC, Area Under Curve; CI, Confidence Interval; pSS, primary Sjögren's syndrome

[a]Cut-off points obtained by maximizing both sensitivity and specificity

values produced a better area under the ROC curve (AUC) than percentages (Table 3; Supplementary Table 2).

The best discriminators were the TMem cells count (in which a cut-off equal to 72 cell/µl had a specificity of 0.75 and a sensitivity of 0.74 for pSS) and the SwM cells subset count (in which a cut-off equal to 35.5 cells/µl had a specificity of 0.79 and a sensitivity of 0.75 for pSS) (Table 3). The former cut-off was fulfilled by 75.4% of pSS patients and 20.8% of controls, whereas 61.2% of Sicca patients also presented SwM counts lower than the cut-off. Regarding the TMem cut-off of 72 cells/µl, it was fulfilled by 73.7% of pSS patients, 25.0% of controls, and 53.7% of Sicca patients.

In the Bm1–Bm5 classification, the item with best performance was the sum of eBm5 with Bm5 populations, in which, using a cut-off value of 38 cells/µl, a specificity of 0.75 and a sensitivity of 0.72 for pSS was obtained (Table 3). That cut-off was fulfilled by 71.9% of pSS patients, 25.0% of controls, and 55.2% of Sicca patients. The ratio Bm2 + Bm2′/eBm5 + Bm5 presented an optimal cut-off value of 3.83, however, with a sensitivity of only 0.53 and specificity of 0.75.

To reduce the chance of false positives, we have tested several specificity values between 0.80 and 0.95 and determined that a 0.88 specificity yielded the cut-offs that best distinguished pSS patients from controls, without severely compromising sensitivity. Overall, slightly better results were obtained with absolute values than with percentages (Table 3; Supplementary Table 2). A cut-off of equal to 58 TMem cells/µl was met by 59.6% of pSS patients, 12.5% of controls and 38.8% of Sicca patients, and a cut-off of equal to 23.5 SwM cells/µl was met by 54.4% of pSS patients, 12.5% of controls and 37.3% of Sicca patients. In the Bm1–Bm5 classification, the eBm5 + Bm5 population was a good discriminator (AUC = 0.77), with a cut-off of 23.33 cells/µl corresponding to 0.88 specificity, and 0.47 sensitivity. Values lower than this cut-off were obtained in 47.4% of pSS patients, 8.3% of controls and 31.3% of Sicca patients.

Comparison with phenotypic features of pSS

Considering the cut-off values for 0.88 specificity established in TMem and SwM B-cell counts, we compared the immunological profile and phenotypic features of pSS patients bellow and above the established cut-off (Table 4).

For both cell populations, patients with cell counts bellow the cut-off were older and had longer disease duration than patients with cell counts above the cut-off, however only the disease duration reached statistical significance (14.3 versus 8.1 years, $p = 0.006$ for TMem, and 13.8 versus 9.4 years, $p = 0.042$ for SwM) (Supplementary Table 3).

Regarding the presence of a focus score ≥ 1 in MSG biopsy, it was more frequent in patients with cell counts below the established cut-off, with an odds ratio of 1.4

for TMem and 1.7 for SwM, although the differences were not statistically significant ($p = 0.618$ and $p = 0.452$, respectively).

Autoantibody occurrence (anti-SSA, SSB, ANA and RF) was higher in the pSS patients group with TMem ≤ 58 or SwM < 23.5 cell/µl, but not reaching statistical significance. Patients with positive anti-SSA were more likely to present TMem or SwM counts below the cut-off, compared to SSA-negative patients, with an odds ratio estimate of 2.1 for TMem and 1.5 for SwM. Similarly, a greater odds ratio estimate was determined for anti-SSB positivity (3.5 for TMem and 2.9 for SwM) and for ANA ≥ 1/320 (2.5 for TMem and 1.9 for SwM).

Although the presence of extra-glandular manifestations did not differ between patients below or above the B-cell cut-offs, patients below the cut-off did had significantly higher ESSDAI score than patients above the cut-off (3.1 versus 1.4, $p = 0.043$ for TMem, and 3.3 versus 1.3, $p = 0.022$ for SwM—Supplementary Table 3). Among the 9 patients with at least moderate disease activity (ESSDAI ≥ 5), 8 presented TMem and SwM cell-counts below the cut-off (with $p = 0.082$ and $p = 0.049$, respectively, compared to patients above the cut-off). By univariate analysis, we determined that patients with ESSDAI ≥ 5 were 6.8 and 8.7 times more likely to have TMem ≤ 58 cells/µl and SwM < 23.5 cells/µl, respectively. Also, patients with gammaglobulin levels above 1.6 g/dl were 3.2 and 2.6 times more likely to have TMem ≤ 58 cells/µl and SwM < 23.5 cells/µl, respectively.

Discussion

B-cell subpopulations defined by the Bm1–Bm5 classification have been previously evaluated as putative diagnostic elements for pSS [30, 31]. To our knowledge, this is the first study that attempts to establish cut-offs for the naïve and memory B-cell subpopulations defined by the IgD/CD27 expression. Therefore, we have assessed the distribution of B-lymphocyte subsets in two groups of patients (pSS and Sicca) and in healthy controls, to determine the cut-off values that better discriminate between pSS and healthy individuals using both gating strategies (IgD/CD27 and Bm1–Bm5 classification with IgD/CD38).

In our study, we have found a significant reduction in the absolute numbers and percentage of UnSwM B-cells in pSS patients compared to controls (in line with the lower number of lymphocytes). These results are in line with those obtained in other studies [20, 24–26, 34], and are likely to represent the impairment of B cell tolerance checkpoints. The mobilization of self-reactive naïve B cells from the bone marrow to the periphery is increased in pSS [20], with migration of peripheral memory B-cells to the affected salivary glands [19, 26] where they account for the majority of

Table 4 Immunological profile and phenotype of pSS patients according to total memory and switched memory cell count cut-off status

	Total memory B-cells				Switched memory B-cells			
	≤ 58 cells/μl (n = 34) (%)	> 58 cells/μl (n = 23)	p value	$\widehat{OR}$ 95% CI	< 23.5 cells/μl (n = 31)	> 23.5 cells/μl (n = 26)	p value	$\widehat{OR}$ 95% CI
Focus score ≥ 1								
Positive	27/33 (81.8)	16/21 (76.2)	0.618	1.40 (0.35, 5.42)	25/30 (83.3)	18/24 (75.0)	0.452	1.67 (0.44, 6.61)
Negative	6/33 (18.2)	5/21 (23.8)			5/30 (16.7)	6/24 (25.0)		
Anti-SSA								
Positive	25/34 (73.5)	13/23 (56.5)	0.185	2.18 (0.70, 6.71)	22/31 (71.0)	16/26 (61.5)	0.453	1.53 (0.50, 4.71)
Negative	9/34 (26.5)	10/23 (43.5)			9/31 (29.0)	10/26 (38.5)		
Anti-SSB								
Positive	14/30 (46.7)	4/20 (20.0)	0.061	3.50 (1.01, 14.49)	13/28 (46.4)	5/22 (22.7)	0.088	2.95 (0.89, 11.02)
Negative	16/30 (53.3)	16/20 (80.0)			15/28 (53.6)	17/22 (77.3)		
ANA ≥ 1/320								
Positive	29/34 (85.3)	16/23 (69.6)	0.160	2.54 (0.70, 9.85)	26/31 (83.9)	19/26 (73.1)	0.324	1.92 (0.53, 7.37)
Negative	5/34 (14.7)	7/23 (30.4)			5/31 (16.1)	7/26 (26.9)		
RF								
Positive	17/31 (54.8)	7/19 (36.8)	0.219	2.08 (0.66, 6.97)	14/28 (50.0)	10/22 (45.5)	0.750	1.20 (0.39, 3.73)
Negative	14/31 (45.2)	12/19 (63.2)			14/28 (50.0)	12/22 (54.5)		
Gamma ≥ 1.6 g/dl								
Positive	11/34 (32.4)	3/23 (13.0)	0.107	3.19 (0.85, 5.58)	10/31 (32.3)	4/26 (15.4)	0.148	2.62 (0.75, 10.75)
Negative	23/34 (67.6)	20/23 (87.0)			21/31 (67.7)	22/26 (84.6)		
EGM								
Positive	15/34 (44.1)	8/23 (34.8)	0.482	1.48 (0.50, 4.55)	15/31 (48.4)	8/26 (30.8)	0.180	2.11 (0.72, 6.50)
Negative	19/34 (55.9)	15/23 (65.2)			16/31 (51.6)	18/26 (69.2)		
ESSDAI ≥ 5								
Positive	8/34 (23.5)	1/23 (4.3)	0.082	6.77 (1.12, 130.65)	8/31 (25.8)	1/26 (4.3)	0.049	8.70 (1.44, 167.82)
Negative	26/34 (76.5)	22/23 (95.7)			23/31 (74.2)	25/26 (95.7)		
Objective ocular signs								
Positive	17/33 (51.5)	18/23 (78.3)	0.047	0.30 (0.08, 0.94)	14/30 (46.7)	21/26 (80.8)	0.011	0.21 (0.06, 0.67)
Negative	16/33 (48.5)	5/23 (21.7)			16/30 (53.3)	5/26 (19.2)		
Objective oral signs								
Positive	18/29 (62.1)	16/19 (84.2)	0.109	0.31 (0.06, 1.19)	17/26 (65.4)	17/22 (77.3)	0.369	0.56 (0.15, 1.96)
Negative	11/29 (37.9)	3/19 (15.8)			9/26 (34.6)	5/22 (22.7)		
Dry eye symptoms								
Positive	32/34 (94.1)	22/23 (95.7)	0.800	0.73 (0.03, 0.73)	29/31 (93.5)	25/26 (96.1)	0.664	0.58 (0.03, 6.41)
Negative	2/34 (5.9)	1/23 (4.3)			2/31 (6.5)	1/26 (3.9)		
Dry mouth symptoms								
Positive	32/34 (94.1)	23/23 (100.0)	0.992	–	29/31 (93.5)	26/26 (100.0)	0.992	–
Negative	2/34 (5.9)	0/23 (0.0)			2/31 (6.5)	0/26 (0.0)		

Odds ratios estimates obtained after discretizing Total Memory and Switched Memory in the two categories: above and below the established cut-offs

Each phenotypic feature is represented as positive and negative cases and corresponding percentages. pSS patients were divided in two groups according to the numbers of total memory (TMem) B-cells (≤ 58 and > 58 cells/μl) and phenotypic features in both groups were compared. The same comparison was performed according to the numbers of switched memory (SwM) B-cells (< 23.5 and > 23.5 cells/μl). A TMem cell count cut-off of 58 cells/μl has a specificity and a sensitivity of 0.88 and 0.60 for pSS, respectively. A SwM cell count cut-off of 23.5 cells/μl has a specificity of 0.88 and a sensitivity of 0.54 for pSS, respectively

CI, confidence intervals; SSA/SSB, Sjögren's syndrome A/B antibody; ANA, antinuclear antibody; RF, rheumatoid factor; ESSDAI, European Sjögren's syndrome disease activity index

infiltrating B-cells. Probably there is also a skew of B-cell differentiation towards plasma cells [24, 35]. Age can be associated with a decrease in naïve B-cells and an accumulation of mature B-cells [36]. However, we have found that patients with lower TMem and SwM B-cell counts were older and had longer disease evolution. The impairment in these particular B-cell subsets may be related to either an increased tissue migration pattern, or a failure in the differentiation of such post-germinal centre B cells.

Although the percentages of SwM and UnSwM cells in each group were equivalent, the pSS group presented significantly lower percentages of UnSwM B-cells compared to the control group. The influence of the evolution time in the distribution of the B cell subpopulations should be approached in future prospective studies.

Cut-off values for two scenarios were obtained, and we have chosen greater specificity over optimal sensitivity and specificity, because our objective was to minimize the chance of false positives, comparing pSS and controls. Better discrimination was obtained with absolute counts rather than percentages. Patients fulfilling the established cut-off for either TMem (≤ 58 cells/µl) or SwM (< 23.5 cells/µl) were more likely to present other phenotypic features of pSS, such as FS ≥ 1, anti-SSA and anti-SSB antibodies, ANA, RF and increased IgG. Although statistical significance was not achieved, probably due to the small size of our population, these results support a strong association between lower memory B-cells and immunologic features of pSS. Whether such B-cell cut-offs could be used in substitution of any of the immunological criteria remains to be clarified. The association of higher disease activity, as determined by the ESS-DAI score, with lower total memory and switched memory B-cells may be indicative of the role of these immunological disturbances in disease severity. Adding to its hypothetical diagnostic utility, the assessment of memory B-cells could have prognostic value.

Our results share similarities with those of Roberts et al. [25], which described significantly lower frequencies of SwM and UnSwM B-cells in pSS. However, their work corresponded UnSwM B-cell with phenotypic features of pSS, whereas in our study we have focused in TMem and SwM. Differences in phenotypic associations between SwM and UnSwM B-cell subsets is yet to be clarified.

The distribution of Bm1–Bm5 cells in our study also confirmed the increase in the Bm2 and Bm2′ populations, and the decrease in the eBm5 and Bm5, although less marked than described by other investigators [30, 31]. The optimal Bm2 + Bm2′/eBm5 + Bm5 ratio cut-offs of $\geq 3{,}8$ in absolute counts and $\geq 3{,}2$ in percentages that we obtained were lower than the cut-off of ≥ 5 obtained by Binard et al. [30] (considering only percentages), and were associated with lower sensitivity and specificity. However, populations' characteristics may contribute to these differences,

namely the older age of our control group (51.1 versus 36.8 years) and the lower occurrence of anti-SSA in our pSS group, which at 66.7% positivity, is lower than the southern Europe prevalence of 71.8% reported in a large data collection [37]. Although the independent association of the Bm2 + Bm2′/eBm5 + Bm5 ratio with a pSS diagnosis was confirmed in another study, the integration of this test in the AECG criteria did not improve the diagnostic performance [31]. Additionally, due to current therapy, our patients could have less active disease, possibly contributing to a lower Bm2 + Bm2′/eBm5 + Bm5 ratio. In fact, a significant percentage of patients was on low-dose steroid therapy (less than 10 mg/day), and although circulating B-cells are less affected by glucocorticoids than T-cells [38], the long-term influence or the cumulative-dose influence on B-cells has not been properly evaluated. Comparing patients with or without therapy or excluding medicated patients would introduce a bias because non-medicated patients would tend to have mild or inactive disease. We must also stress out that we aimed to have a study that could represent the daily clinical basis.

In our study, Sicca patients presented lymphocyte populations' counts with values in between those obtained for pSS and controls. Such patients constitute a diagnostic challenge, because many of their clinical characteristics overlap with pSS but they lack the specific immunologic markers for pSS, namely the anti-SSA antibodies and the focal lymphocytic infiltrates in the salivary glands. Considering the IgD/CD27 classification, it is interesting to note that the percentage of naive and memory B-cells in Sicca patients was similar to pSS and different from controls, while their absolute numbers were superior to pSS, but notably lower than controls. This intermediate lymphocyte profile probably indicates the presence of disturbed immunological mechanisms in these individuals, which may have milder forms of the disease or be in an earlier stage. In our Sicca group, about 40% of patients had either TMem or SwM cell count below the established threshold, and we can therefore admit that some of these individuals may effectively have pSS. Roberts et al. [25] have also evaluated Sicca patients and described lower memory B-cells percentages compared to controls, and a subset of Sicca patients with a pSS-like transcription profile. In our study, however, more significant differences were obtained in absolute counts of memory B-cells than in their percentages.

We acknowledge some limitations of our study, related to the composition of the patient groups, since B-cell populations' distribution may be influenced by age, duration of disease and past or current therapy. Our pSS and Sicca patient's groups were constituted of individuals recruited in a clinical setting, therefore including patients with a broad range of age and disease duration, as well as variable disease severity.

Although we tried to minimize the age difference between healthy controls and patients, their difference was still significant. Major age differences between groups could affect classification cut-offs. Nevertheless, the age difference between our groups did not prove to be relevant for the interpretation of our results.

Regarding the effect of therapies in the B-cell subset distribution, in a clinical setting it could only be ascertained in prospective studies or clinical trials. The study of B-cell subsets in non-medicated patients in a clinical setting like ours would probably have a trend towards the selection of mild cases not needing therapy, which would limit the validity of our results. It would be feasible in a triage setting, although there would probably exist an over-representation of early-disease patients, whose B-cell populations' distribution may not be similar to those of more evolved patients.

The criteria used for patient inclusion may pose an additional dilemma, since the use of the classification criteria could fail to identify some patients. However, the use of the "gold standard" clinical diagnosis may increase subjectivity and decrease the reproducibility of the results. Nevertheless, the evaluation of the discriminatory role of B-cell subpopulations in the classification of pSS should be ascertained by comparison with the "gold standard" that is the clinical diagnosis. For that purpose, pSS and Sicca patients not fulfilling pSS classification criteria should be subjected to an expert-driven diagnostic re-evaluation, to identify true pSS based on clinical opinion, and B-cell subsets must be compared in both "clinical" and "classification"-determined pSS patient groups.

B-cell subpopulations assessment by flow cytometry is a non-invasive procedure that is reproducible and easy to perform.

We have demonstrated that in pSS, the presence of lower memory B-cells counts was associated with a longer disease duration and a more active disease, which could represent a possible role as prognostic markers. Therefore, we aim to continue the present study in the future to include more patients, allowing us to clearly prove that these measurements may be used as clinical biomarkers for the follow-up of these patients. In fact, we have clearly demonstrated the chance to identify a cut-off point in these cell populations that can be used to clearly distinguish healthy controls from patients. Additionally, they might also be useful for the early diagnosis of pSS in Sicca patients. Considering the association of lower memory B-cell counts with typical pSS phenotypic features such as hypergammaglobulinemia and antibody occurrence, B-cell subpopulations assessment in Sicca patients could potentially identify individuals in higher risk of progression to pSS.

It remains to be clarified if decreased memory B-cells could complement the immunological items currently used for pSS diagnosis. If so, future classification criteria integrating such items could identify patients presently unclassifiable as pSS and allow their access to novel therapies and clinical trials.

Compliance with ethical standards

Conflict of interest The authors have declared no conflicts of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable 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). All patients have signed an informed consent to participate according to the Declaration of Helsinki.

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