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T follicular-like helper cells in the peripheral blood of patients with primary Sjögren's syndrome

K. A. Brokstad[1] | M. Fredriksen[1] | F. Zhou[2] | B. Bergum[1] | J. G. Brun[3] | R. J. Cox[2,4,5] | K. Skarstein[6]

[1]Broegelmann Research Laboratory, Department of Clinical Science, University of Bergen, Bergen, Norway

[2]The Influenza Center, Department of Clinical Science, University of Bergen, Bergen, Norway

[3]Department of Rheumatology, Haukeland University Hospital, Bergen, Norway

[4]Jebsen Centre for Influenza Vaccine Research, University of Bergen, Bergen, Norway

[5]Department of Research and Development, Haukeland University Hospital, Bergen, Norway

[6]Gade Laboratory for Pathology, Department of Clinical Medicine, University of Bergen, Bergen, Norway

Correspondence
K. A. Brokstad, Broegelmann Research Laboratory, Department of Clinical Sciences, University of Bergen, Haukeland University Hospital, Bergen, Norway.
Email: karl.brokstad@uib.no

Abstract

Primary Sjögren's syndrome (pSS) is a chronic autoimmune disease characterized by exocrine gland dysfunction, mainly causing sicca symptoms. B cells have a prominent role in SS, and the T follicular helper (T_{FH}) cells provide B cells with survival and specialization signals in germinal centres. Here, we investigate peripheral T_{FH} cells in pSS. Sixteen pSS patients and healthy controls were enrolled in the study, with 13 women and 3 men in each group. Whole blood was collected and separated into PBMC and plasma, followed by cryopreservation. Plasma samples were analysed for Ro52, Ro60 and La48 autoantibodies by indirect ELISA. For flow cytometric analysis, we defined 4 subsets of TFH-like cells within the $CD3^+CD4^+CXCR5^+$ population, namely the $ICOS^-PD\text{-}1^-$, $ICOS^-PD\text{-}1^+$, $ICOS^+PD\text{-}1^-$ and $ICOS^+PD\text{-}1^+$ ("TFH") cells. We also investigated 4 $CD19^+$ B cell subsets, the $CD20^+CD27^+CD38^-$ memory B cells, $CD20^+CD27^+CD38^+$ memory B cells, $CD20^-CD27^+CD38^{++}CD138^-$ plasmablasts and $CD20^-CD27^+CD38^{++}CD138^+$ plasma cells. We observed higher fractions of $ICOS^+PD\text{-}1^-$ cells, $ICOS^+PD\text{-}1^+$ ("T_{FH}") cells and plasmablasts in pSS patients compared to controls, and lower frequencies of both types of memory B cells. The number of T_{FH} cells correlated positively with the levels of plasmablasts and plasma cells in the pSS patients, but not in the controls. The pSS patients were stratified according to Ro52/Ro60/La48 serology, and a positive association was found between autoantibody levels and increased level of T_{FH} cells, plasmablasts and plasma cells and lowered levels of memory B cells. We observed a higher response to Ro/La stimulation in pSS patients compared to controls of the memory B cells, although only significantly for the $CD38^-$ memory B cells. Overall, a pathological relation between the $ICOS^+$ T follicular-like helper cells and B cells in pSS was observed, but further work should be conducted to explore their overall impact upon disease progression.

1 | INTRODUCTION

Sjögren's syndrome (SS) is a chronic rheumatic autoimmune disease characterized by impaired exocrine glandular function. This is due to infiltration by mononuclear cells, primarily affecting salivary and lacrimal glands, causing dryness of eyes (keratoconjunctivitis sicca) and mouth (xerostomia).[1] Other common symptoms of SS patients are general signs of inflammation, such as muscle and joint aches, mild fever and fatigue.[2,3] SS can be organ-specific, where the disease is limited to distinct organs (like salivary and lacrimal glands), or systemic, involving extra-glandular

manifestations (commonly skin, lungs and peripheral nerves). Auto-antibodies against the Ro/SSA and La/SSB antigens are commonly found in SS patients and are an important diagnostic indicator of the disease.

The T follicular helper cells (T_{FH}) make up a subpopulation within the T helper (T_H) cells that reside in the B cell area of the secondary lymphoid organs. T_{FH} cells are important in the germinal centre reaction where they assist B cells in isotype switching, somatic hypermutation and affinity maturation.[4] The T_{FH} cells express a combination of molecules that are crucial for their development and function. This includes high levels of the chemokine receptor CXCR5, inducible T cell costimulator (ICOS), Programmed cell death protein-1 (PD-1), CD40 ligand (CD40L), 0X40, SLAM-associated protein (SAP), the transcription factors Bcl-6 and c-Maf and production of the cytokine IL-21.[4] Expression of the B cell zone homing chemokine receptor CXCR5 and down-regulation of the T cell zone homing chemokine receptor CCR7 enables T_{FH} cells to reside in the B cell zone where they execute their function.[5] The interaction between ICOS and its ligand B7-related protein-1 (B7RP-1), expressed on antigen presenting cells (APCs), is essential for optimal B cell differentiation and heavy chain isotype switching.[6,7] Furthermore, Crotty et al.[8] suggested that the formation of plasma cells and T effector memory cells are dependent on SAP molecule which is expressed on the T_{FH} cells. Interestingly, mutations in this gene have been linked to hypogammaglobulinemia.[9] The transcription factor B cell lymphoma 6 (Bcl-6) is the main T_{FH} cell regulator and is largely confined to the germinal centre T and B cells.[4] In activated T cells, the c-Maf induces production of IL-21, which is a cytokine crucial for B cell proliferation and differentiation.[10] CD40L is critical for germinal centre B cell survival,[4] whilst PD-1 inhibits T cell signalling in germinal centres and thereby prevents excessive antibody responses.[11] Traditionally, the T_{FH} cells were mainly defined by their anatomical location, but recent studies have revealed the existence of peripheral analogues. A key difference between the stationary and circulating T_{FH} cells is that the latter lack expression of Bcl-6.[12]

The objective of this study was to investigate T_{FH}-like cells and study their possible pathological relationship to B cell subsets, ie memory B cells, plasmablasts and plasma cells, in peripheral blood of pSS patients. Additionally, the pathological relationship was related to clinical findings in the pSS patients and Ro/La autoantibodies.

2 | MATERIAL AND METHODS

2.1 | Patients and controls

Sixteen primary Sjögren's syndrome patients and 16 controls where enrolled in this study, including 13 women and 3 men in both groups. The patients were recruited from the Rheumatology Clinic at Haukeland University Hospital (HUH), Bergen, Norway. All patients fulfilled the American European Consensus Group (AECG) criteria.[13] for Primary Sjögren's syndrome (Table 1). Demographics and clinical data were extracted from medical records and provided by the Department of Rheumatology at HUH. The patients' mean age was 60 years (range: 29-85 years). Control volunteers above 40 years of age were recruited at the Blood Bank at HUH, with a mean age of 52 years (range: 40-65 years). All patients and controls received written and oral information, and participating patients provided a signed consent. The study has been approved by the regional ethical committee (REK.no: 3.2006.3085).

2.2 | Sample collection, handling and storage

Samples were collected in the period September to late November 2015. Peripheral blood was drawn in heparin tubes and processed within 1 hour of sampling. The whole blood was diluted 1:2 with PBS, and separated by density gradient centrifugation (800 g, 20 minutes, 22°C, brake off) using Lymphoprep (LYS3773; Axis-Shield, UK) into plasma and mononuclear cell (PBMC) fractions. The plasma was aliquoted and stored at −70°C. The PBMCs were resuspended in freezing medium (FM; 20% DMSO, D5879-500ML; Sigma-Aldrich, USA, 80% FBS; PAA A15-151; PAA laboratories, USA) and frozen in a freezing container, giving a controlled cooling rate of −1°C/min down to −70°C, minimizing the risk of crystal formation. The cells were stored in a liquid nitrogen container.

2.3 | Plasma—ELISA

The plasma levels of autoantibodies against Ro52, Ro60 and La48 was performed as described in a previous publication.[14] Briefly; Microlon medium binding plates were coated with 0.1 μg/100 mL/well recombinant autoantigen. For all our studies, we have used affinity purified full length human recombinant Ro52, Ro60 and La48 as described in Garberg et al.[14]

Fetal Calf Serum (FCS) was used to block unbound areas, and extensive washing was performed between each step (PBS with 0.5% Tween). The plasma was added to the wells in duplicate at 1:1000 dilution. Detection antibody was goat-anti-human-IgG POX conjugated (A0293; Sigma-Aldrich) and using the OPD substrate. The plates were read at OD 492 nm with a Biotek Synergy H1 microplate reader. The cut-off value for a positive serology was set to the mean optical density (OD) of the control subjects plus 2 standard deviations (SD). The cut-off values were calculated to 0.120, 0.230 and 0.150 for Ro52, Ro60 and La48, respectively (Figure S1).

TABLE 1 Demographic and clinical data of the patients

				Clinical features						
						Autoantibodies[b]				
Patient	Gender (M/F)	Age (y)	Clinical diagnose (y)	ESSPRI	Focus score[a]	ANA	SSA	SSB	ACPA	RF[c]
1[d]	M	85	2002	6.67	1	+	+	–	+	493
2	M	54	2014	6.67	3	+	+	+	–	328
3	F	70	2003	8.33	1	+	+	–	–	<11
4	F	53	1997	7.33	1	+	+	–	–	<11
5	F	78	2005	8.67	N/D[e]	+	+	–	–	41
6	F	58	2010	7.33	N/D	+	+	+	–	215
7	F	58	2015	7.0	1	–	–	–	N/D	N/D
8	F	64	1997	9.0	1	+	+	–	–	<11
9	F	82	1992	7.33	2	–	–	–	–	<11
10	F	37	2011	6.0	N/D	+	+	+	N/D	N/D
11[d]	F	72	2004	5.0	0	(–)	(–)	–	–	<11
12	M	73	2003	4.67	N/D	+	+	+	–	<11
13	F	58	2010	4.67	N/D	–	–	–	–	<11
14	F	48	2014	7.33	2	+	+	+	N/D	N/D
15	F	29	2009	3.33	1	+	+	–	–	11
16	F	41	2006	6.33	N/D	+	+	–	–	<11

[a]Focus score: the number of foci containing at least 50 mononuclear cells per 4 mm^2 of glandular tissue.
[b]SSA: anti-Ro60 and/or anti-Ro52. SSB: anti-La48. The +/– indicates a positive/negative serology and () indicates weak results.
[c]RF values are given as IU/mL.
[d]It took more than 1 h from blood sampling to the first centrifugation (here: approximately 3 h).
[e]N/D: No data.

2.4 | Cells—flow cytometry

Cell growth conditions: LM: RPMI 1640, BE 12-702F/U1, Lonza, Belgium, +10% FBS +1% Penicillin, Streptomycin and Amphotericin B, 17-745E, Lonza. AM: LM + 1 μg/mL Mouse anti-human CD28, 1 μg/mL Mouse anti-human CD49d. Ro/La: AM + Ro52/Ro60/La48 cocktail 1 μg/mL of each autoantigen. PHA: AM + 1 μg/mL PHA-L, L4144, Sigma-Aldrich. The frozen PBMC samples were rapidly defrosted in prewarmed (37°C) lymphocyte medium (LM) and washed multiple times in LM. Finally, the cells were suspended in 1 mL of LM and counted using Casyton (05651808; Roche Innovatis, Germany), and the cell suspension was adjusted to 1×10^7 cells/mL. PBMCs were transferred to a 96-well tissue culture plate, with a 100-μL cell suspension (1×10^6 cells) in each well. Cells were incubated in either lymphocyte medium (LM), activation medium (AM), AM with Ro/La, (Ro/La) or AM with Phytohaemagglutinin (PHA) for 16 hours (37°C, 5% CO_2) in duplicate. After stimulation, the cells were stained with first Live/Dead stain solution (Table S1), for 30 minutes at 4°C, followed by 50 μL Fc-block solution (10% human serum/FM) for 15 minutes at 4°C, and lastly the fluorochrome-conjugated antibody panel (Table S1), for 30 minutes at 4°C. After washing the cells, they were resuspended and transferred to microtitre tubes using 350 μL FM and analysed by flow cytometric analysis (BD LSR FortessaTM Cell Analyser; BD Biosciences, USA). The gating strategy is illustrated in Figures S2-S4.

2.5 | Statistical analysis

Graphs and Statistics were performed using Prism for Mac OS X v.5.0f (GraphPad Software). The significance of the results was determined using Welch′s unequal variances *t* test when comparing pSS patients with controls. Non-parametric-paired *t* test was used when comparing the effect of various stimulation media on subsets within the same subject group. *P*-values <.05 were considered significant.

3 | RESULTS

In this study, samples from 16 age and sex matched pSS patients and controls were analysed for Ro/SSA and La/SSB autoantibodies and flow cytometry was used to quantify T_{FH}-like cells and B cell subsets (ie memory cells, plasmablasts and plasma cells).

3.1 | Serological detection of autoantibodies against Ro52, Ro60 and La48

Indirect ELISA was conducted to determine the levels of plasma autoantibodies against Ro52, Ro60 and La48. All the controls had anti-Ro and La serology deemed negative. There were significant differences between pSS patients and controls for Ro52 (P = .0003), Ro60 (P = .0045) and La48 (P = .0234; Figure S1). The results from in-house ELISA were compared with the data from the routine diagnostic lab at the Rheumatology Clinic (Table S2). Only 4 pSS patients showed inconsistent serology results between the in-house analysis and the test performed at the clinical routine laboratory.

3.2 | Flow cytometry

Plasma and mononuclear cells from pSS patients and control subjects were either left unstimulated or exposed for 16 hours in one of the following stimulation media; activation media (AM), recombinant Ro/La cocktail (1 μg/mL) in AM or PHA (1 μg/mL) in AM as a positive control. The cells were then fixed and stained with antibody panels, identifying T_{FH}-like cells and B cell subsets followed by flow cytometric analysis. The proportion of $CD4^+$ cells in the $CD3^+$ cell pool was stable in pSS patients (60%) and controls (67%) under all conditions tested (Figure 1A), and the difference was not statistically significant. Testing with hierarchical linear regression analysis revealed that age did not contribute to the difference in the $CD4^+$ population, and the slightly lower $CD4^+$ counts in patients can be attributed to the disease state (data not shown). A significant increase cell count for all stimulated cells in both patient and control samples was found in $CD3^+CD4^+CXCR5^+$ cells (Figure 1B).

3.3 | Levels of T_{FH}-like cells in pSS

Four subsets of T_{FH}-like cells were identified within the $CD3^+CD4^+CXCR5^+$ gating; $ICOS^-PD\text{-}1^-$, $ICOS^+PD\text{-}1^-$, $ICOS^-PD\text{-}1^+$ and $ICOS^+PD\text{-}1^+$ ("T_{FH}") cells. In this paper, these are referred to as T_{FH}-like cells, with the $ICOS^+PD$-

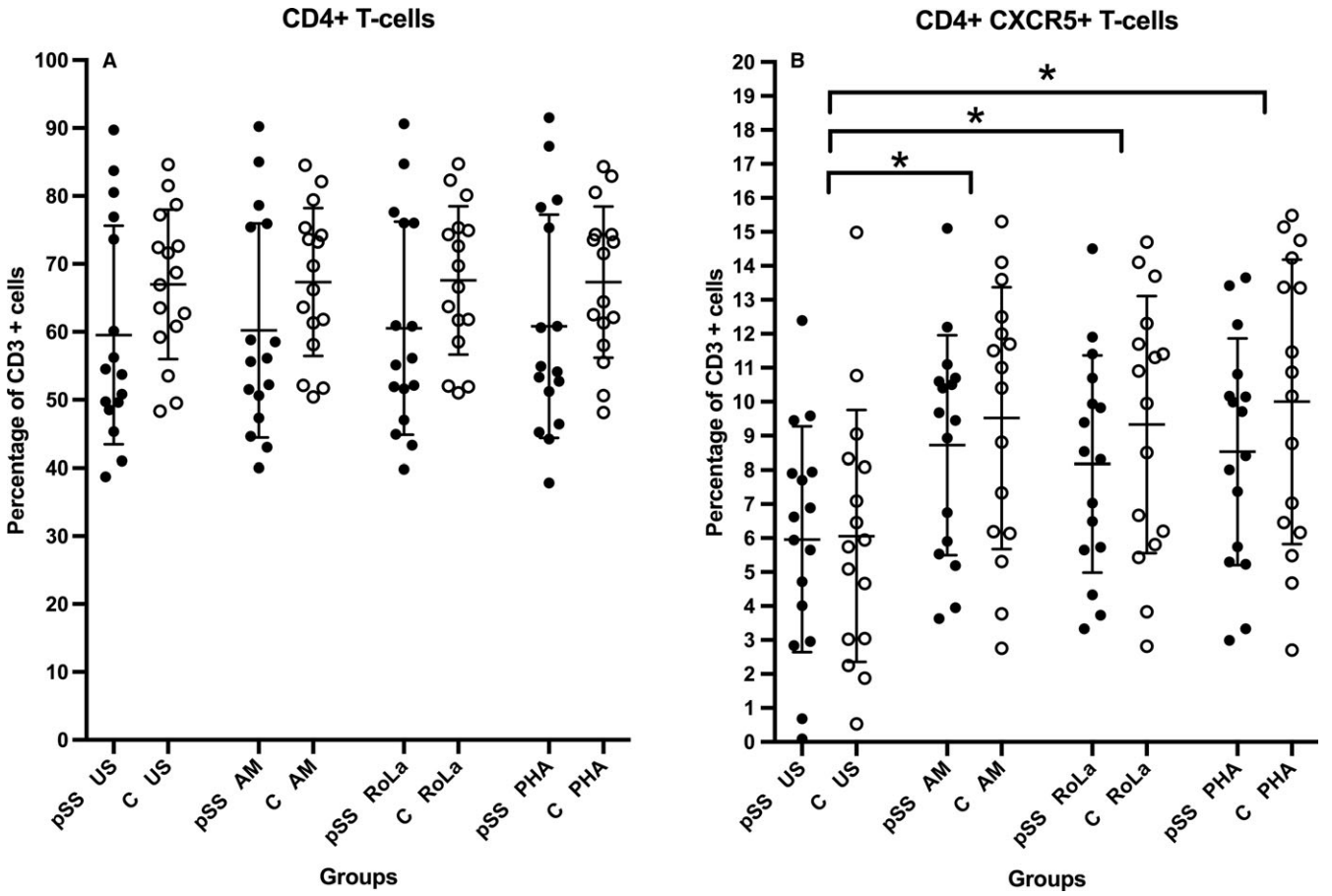

FIGURE 1 The proportion of $CD4^+$ and $CD4^+/CXCR5^+$ cells of the $CD3^+$ gated cells. On the x-axis, the pSS patient and control samples are grouped according to culture conditions (pSS = patients; C = controls; US = unstimulated; AM = activation medium; RoLa = Ro/La stimulation; PHA = phytohemagglutinin stimulation). The y-axis shows the percentage of $CD4^+$ of $CD3^+$ cells. Each symbol represents one subject, with close circles being the patients and the open being the controls. The horizontal lines indicate the mean and ± standard deviation (SD).

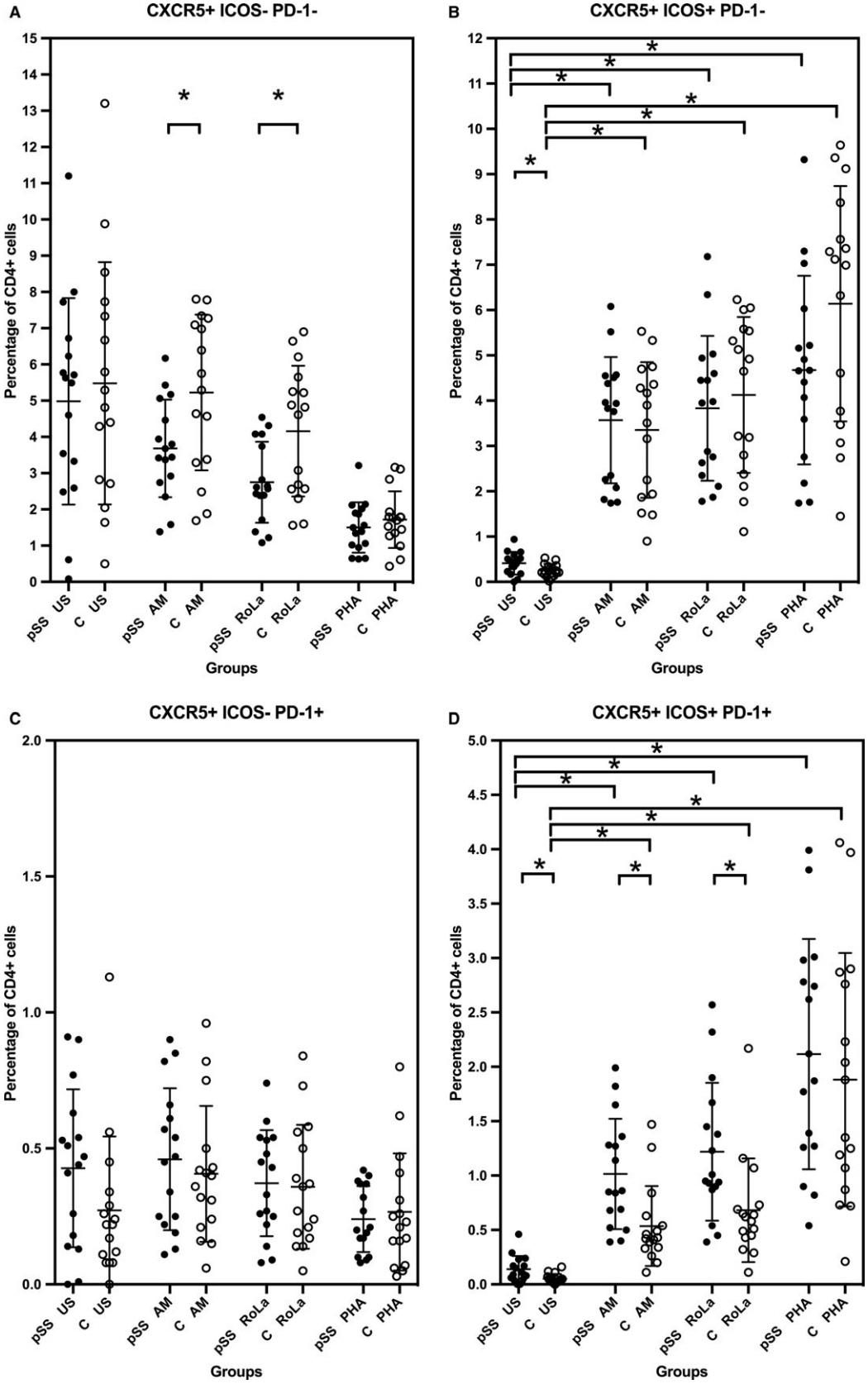

FIGURE 2 The distribution of $CD3^+CD4^+CXCR5^+$ cells expressing ICOS and PD-1 (TFH-like cells). The samples are sorted according to subject group (C or pSS) and culture condition (US, AM, RoLa or PHA), ICOS and PD-1 expression is shown in the upper legend and presented as the fraction of $CD4^+$ cells within the $CD3^+$ gating. The lines represent mean±SD and significant results ($P < 0.05$) of interest are marked with an asterisk (*).

1^+ population being most similar to the T_{FH} cells in the GC. Figure 2 shows the proportion of T_{FH}-like cells under various culture conditions. The proportion $ICOS^-PD\text{-}1^-$ cells (Figure 2A) were significantly lower in the patients compared to the controls when the cells were AM cultivated ($P = .0223$) and RoLa-stimulated ($P = .0135$). The same tendency was seen with the unstimulated (mean ± SD; pSS = 5.0 ± 2.8%, C = 5.5 ± 3.3%), and PHA stimulated (pSS = 1.5 ± 0.7%, C = 1.7 ± 0.8%) samples but the differences were not significant. The fraction of $ICOS^-PD\text{-}1^-$ population decreased with stimulation with RoLa ($P = .0087$) and PHA ($P = .0002$) for patients. A significantly higher level of $ICOS^+PD\text{-}1^-$ cells (Figure 2B) was observed in pSS patients (0.4 ± 0.2%) compared to controls (0.3 ± 0.1%; $P = .0397$), in unstimulated samples. The proportion increased significantly from the AM-stimulated to the RoLa-stimulated samples for both groups (C $P = .0009$, pSS $P = .0214$). The number of $ICOS^-PD\text{-}1^+$ cells decreased slightly with stimulation (Figure 2C), and there was no significant difference between the pSS patients and controls. The proportion of ICOS+PD-1+ ("T_{FH}")- cells was significantly higher within the patient group compared to the controls, in the unstimulated ($P = .0136$), AM-stimulated ($P = .0049$) and RoLa-stimulated ($P = .0116$) samples (Figure 2D). In unstimulated samples, the level of "T_{FH}"-cells was 0.14 ± 0.12% (mean ± SD) for the pSS patients, and 0.05 ± 0.04% for the control subjects, indicating a 2.8-fold difference. The proportion was significantly higher in the RoLa-stimulated samples compared to the AM-stimulated samples for both groups (C; $P = .0026$, pSS; $P = .0084$).

3.4 | Levels of B cell subsets in pSS

The total level of B cells ($CD19^+$) when cultured in activation medium (AM) with and without RoLa or PHA, did not increase significantly in either patients or controls (Figure 3).

Four subsets of B cells were of particular interest, namely $CD38^-$ memory B cells, $CD38^+$ memory B cells, plasmablasts and plasma cells using the following markers to distinguish the cell subsets; CD19, CD20, CD27, CD38 and CD138 (Figure 4).

Controls had significant higher $CD38^-$ memory cells between patients and controls in all 4 groups of stimuli, where controls had consistently higher levels (US $P = .0003$, AM $P = .0013$, RoLa $P = .0010$, PHA $P = .0157$). The level of $CD38^-$ memory cells decreased significantly in PHA-treated cells compared to the unstimulated groups (pSS $P = .0047$, C $P = .0317$), but not for the other treatment groups. The level of $CD38^-$ memory cells was 8.3 ± 5.8% in the patients and 22.8 ± 12.3% in the controls in unstimulated samples, revealing a 2.7-fold higher level of $CD38^-$ memory cells in the controls.

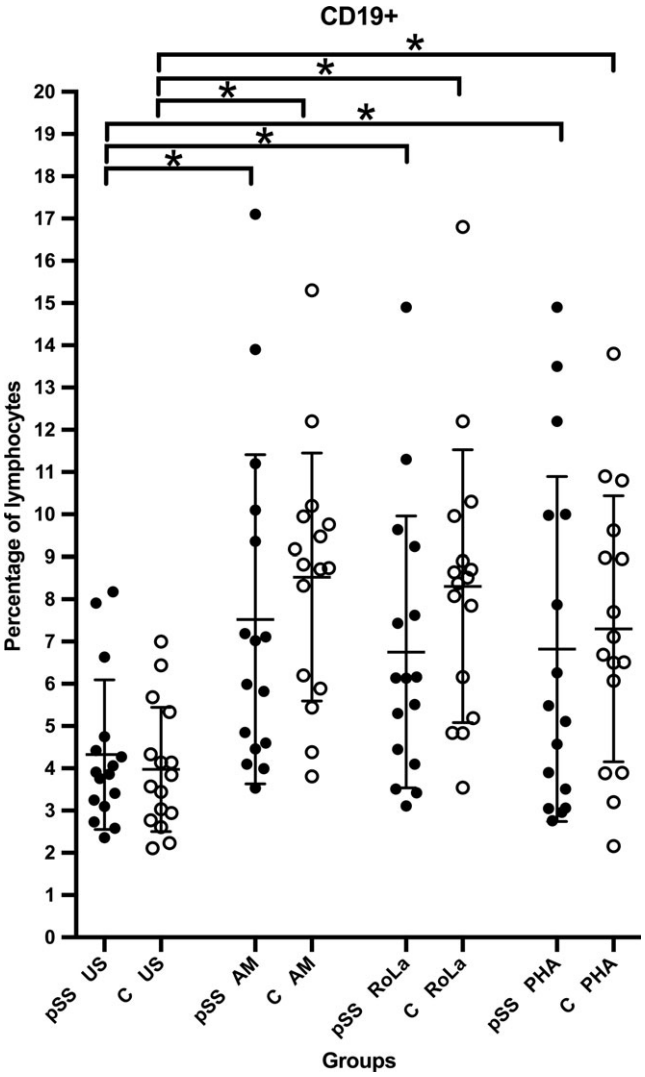

FIGURE 3 Distribution of B cells ($CD3^-$ $CD19^+$) of the total lymphocyte population. Each column is grouped into patients (pSS) and controls (C), and growth condition (US, AM, RoLa and PHA). Each individual sample is indicated by a closed circle (pSS patients) and open circle (controls). The lines show the mean ± standard deviation (SD). The patients and sample treatment are shown under the *x*-axis, and the percentage of B cells out of the total lymphocyte population on the *y*-axis. * denotes statistically significant changes ($P < 0.05$).

The fraction of $CD38^+$ memory B cells was significantly lower within the patient group compared to the controls under all the tested conditions (Unstim $P = .0065$, AM $P = .0443$, RoLa $P = .0163$, PHA $P = .0112$). In unstimulated cell samples, the level of $CD38^+$ memory cells was 11.8 ± 4.9% for the pSS patients and 16.9 ± 5.0% for the controls, 1.4-fold higher in controls.

An increased level of plasmablasts was observed in unstimulated samples of pSS patients (1.9 ± 1.8%) compared to controls (0.9 ± 0.6%; $P = .0490$). This difference was also observed between patients and controls in AM

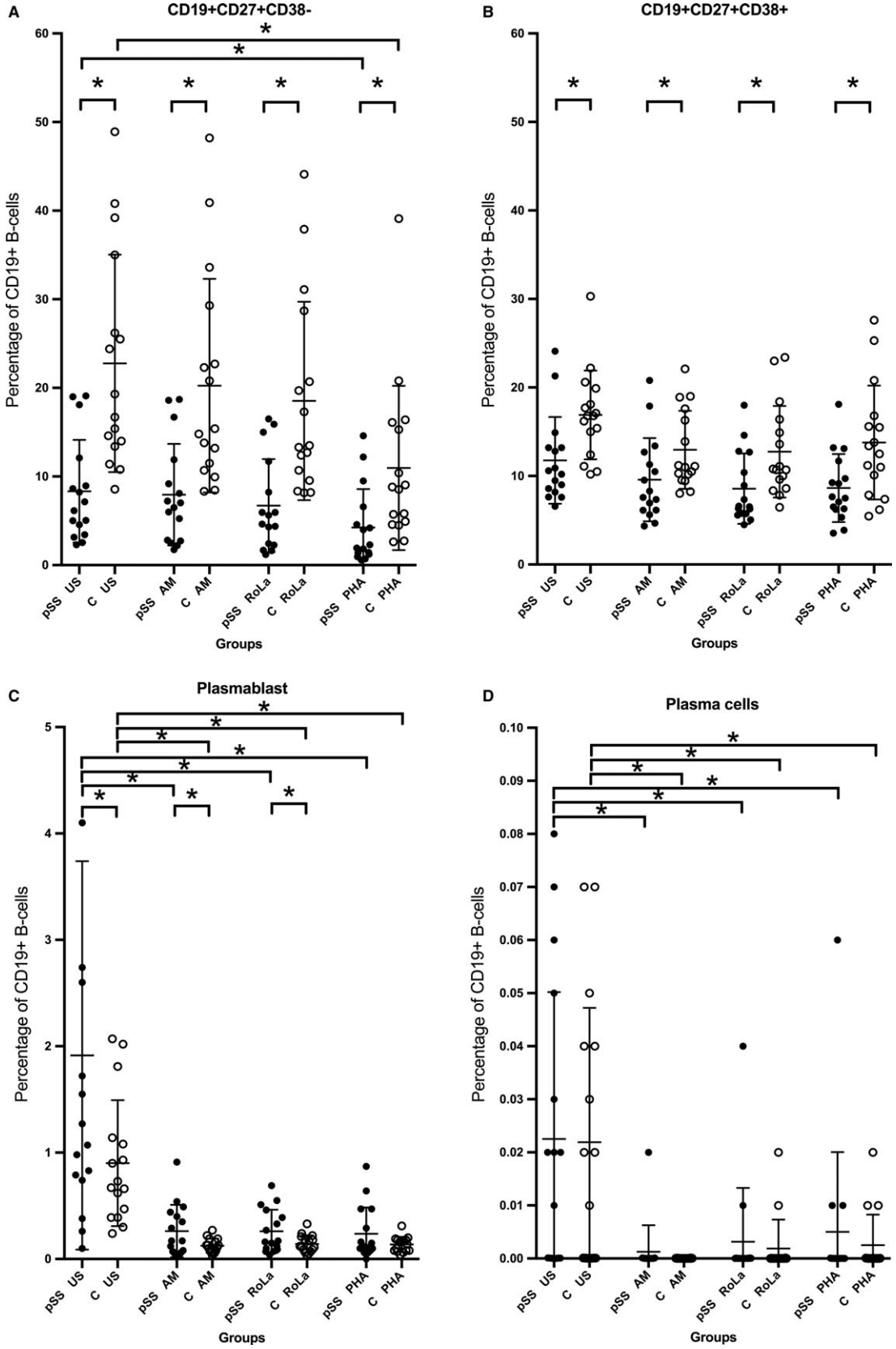

FIGURE 4 B cell subsets; levels of memory cells, plasma blasts and plasma cells in the $CD3^{-}CD19^{+}$ cell population. The four panels show the $CD38^{-}$ and $CD38^{+}$ memory B cells, plasma blasts and plasma cells as indicated in the legend on the top of each panel. The columns show the samples grouped into patients and controls (pSS and C), and treatment (US, AM, RoLa and PHA). Each circle indicates one individual sample, and the horizontal lines is the mean value ± standard deviation (SD). * denotes statistically significant differences ($P < 0.05$).

(P = .0447) and RoLa (P = .0414). The level of plasmablasts dropped when cultivating with AM, RoLa and PHA in both the patient (P = .0026, P = .0025, P = .0023) and control groups (P = .0001, P = .0001, P = .0001) compared to unstimulated cells.

No difference was observed between patients and controls in plasma cell numbers, but the levels dropped significantly in the cultivated cells groups; AM (P = .0081 and P = .0036), RoLa (P = .0165 and P = .0069) and PHA (P = .0362 and P = .0085) for both patients and controls, respectively.

3.5 | Levels of T_{FH}-like cells in pSS patients grouped according to Ro/La serology

The pSS patients were stratified into groups according to Ro/La serology results from indirect ELISA (Table S3 and Figure 1); serum negative (−, n = 3), single positive (+, n = 2), double positive (++, n = 4) and triple positive (+++, n = 7) subjects against Ro52, Ro60 and La48. Figure 5 illustrates the proportion of T_{FH}-like cells under various conditions sorted by the Ro/La serology profile.

In general, the $ICOS^-$ subsets decreased and the $ICOS^+$ subsets increased with in vitro stimulation, as also seen in Figure 2. The populations $ICOS^-PD\text{-}1^-$, $ICOS^+PD\text{-}1^-$ and $ICOS^-PD\text{-}1^+$ did not reveal any statistical difference between the stratified groups. A significantly elevated level of $ICOS^+PD\text{-}1^+$ "T_{FH}"- cells was found in triple positive compared to negative and single positive subjects.

A trend of falling numbers of $CD38^-$ memory B cells from serum negatives to triple positives was observed under all tested conditions (Figure 6), with a significant difference between serum negatives and triple positives under all conditions. Although when stratifying patients with respect to Ro/La serology, we were not able to observe any changes in $CD38^+$ memory cell levels.

The levels of both plasma cells and plasmablasts were more prominent with stronger Ro/La serology (Figure 7). The plasmablast levels were 0.4 ± 0.4% in serum negative patients and 2.6 ± 1.6% in triple positive patients (P = .0117). For plasma cells the levels were 0.005 ± 0.004% and 0.03 ± 0.02%, respectively (P = .0094).

3.6 | Comparison of pSS patients according to the level of "T_{FH}"-cells

The pSS patients were divided into 2 groups reflecting their level of $ICOS^+PD\text{-}1^+$ ("T_{FH}") cells. Patients expressing an elevated level of "T_{FH}" cells were defined as "T_{FH}"-hi (n = 7), while those being under average were defined as "T_{FH}"-lo (n = 9; Figure 8). Figure 8 shows the proportion of B cell subsets in unstimulated samples sorted after "T_{FH}"-profile, and linear correlation plots regarding levels of plasmablasts and plasma cells in relation to "T_{FH}" level. No associations were established between B cell subsets and "T_{FH}" cells in control subjects (not shown).

The number of "T_{FH}" cells in pSS patients correlated with the level of plasmablasts (P = .0251) and plasma cells (P = .0029; Figure 8, panels C and D). Considering "T_{FH}"-hi vs "T_{FH}"-lo individuals, the same association was found (PB P = .0248, PC P = .007). The levels of plasmablasts were 3.2 ± 2.1% and 0.9 ± 0.6%, respectively, indicating a 3.6-fold difference. As concerns the plasma cells, the levels were 0.05 ± 0.03% and 0.01 ± 0.01%, suggesting a 5-fold difference.

No significant differences were found between the 2 groups looking at the memory B cells (MC $CD38^-$ P = .1428, MC $CD38^+$ P = .3291). However, the "T_{FH}"-hi groups showed a significant higher level of $CD38^+$ MCs compared to $CD38^-$ MCs (P = .0326). This association was not found with the "T_{FH}"-lo group (P = .7344).

3.7 | Comparison of pSS patients sorted by clinical data

The pSS patients were stratified according to clinical features, ie focus score (FS < 2 n = 7, FS ≥ 2 n = 3), rheumatoid factor (RF ≤ 11 n = 9, RF > 11 n = 4), autoantibodies (ANA/SSA− n = 4, ANA/SSA+ n = 12) and disease activity score (ESSPRI ≤ 5 n = 4, ESSPRI > 5 n = 12; Table 1). Figure 9 shows the proportions of investigated B cell subsets and T_{FH}-like cells in unstimulated samples sorted accordingly.

Regarding T_{FH}-like cells, the levels were similar comparing the subgroups of RF and ESSPRI (ie RF ≤ 11 vs RF > 11 and ESSPRI ≤ 5 vs ESSPRI > 5), whilst there was a general trend of higher levels in ANA/SSA+ patients compared to ANA/SSA− patients. However, the difference was only significant for the $ICOS^+PD\text{-}1^+$ group (P = .0247).

Regarding the memory B cells, the same trend was observed when sorting by ANA/SSA and ESSPRI—the groups with clinical features indicating increased disease activity (ie ANA/SSA+ and ESSPRI > 5) had decreased levels of $CD38^-$ MCs and raised levels of $CD38^+$ MCs. Even so, the difference was only significant for $CD38^-$ MCs comparing the subdivisions of ANA/SSA (P = .0030). In terms of rheumatoid factor, the patients with RF > 11 showed tendencies of lowered levels of both $CD38^-$ and $CD38^+$ MCs, although not significant.

The pSS patients with indications of highest disease activity (ie RF > 11, ANA/SSA+, ESSPRI > 5) had higher levels of $CD38^+$ MCs compared to $CD38^-$ MCs, although not significant for the RF subdivision (RF > 11 P = .1250, ANA/SSA+ P = .0005, ESSPRI > 5 P = .0269). For those with indications of lowest disease activity, this was also

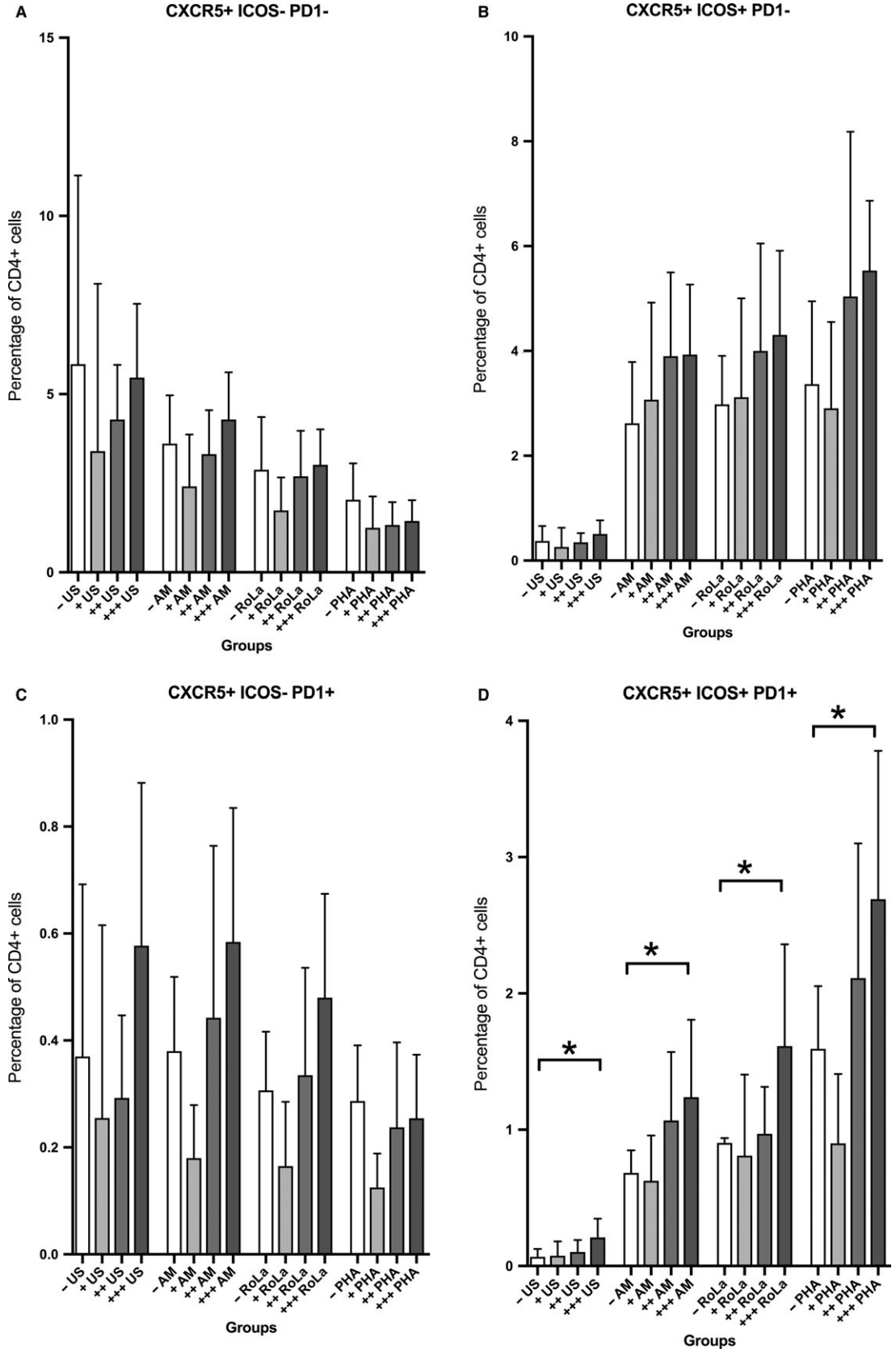

FIGURE 5 $CXCR5^+$ T cells stratified by Ro and La serology. The four panels show the four subsets of Tfh-like T cells based on ICOS and PD-1 expression. The patient samples are divided into Ro52, Ro60 and La48 serology (– = negative, + = single positive, ++ = double positive, +++ = triple positive) and culture conditions (US, AM, RoLa and PHA). Each bar indicates mean level ± SD. *denotes statistically significant differences ($P < 0.05$).

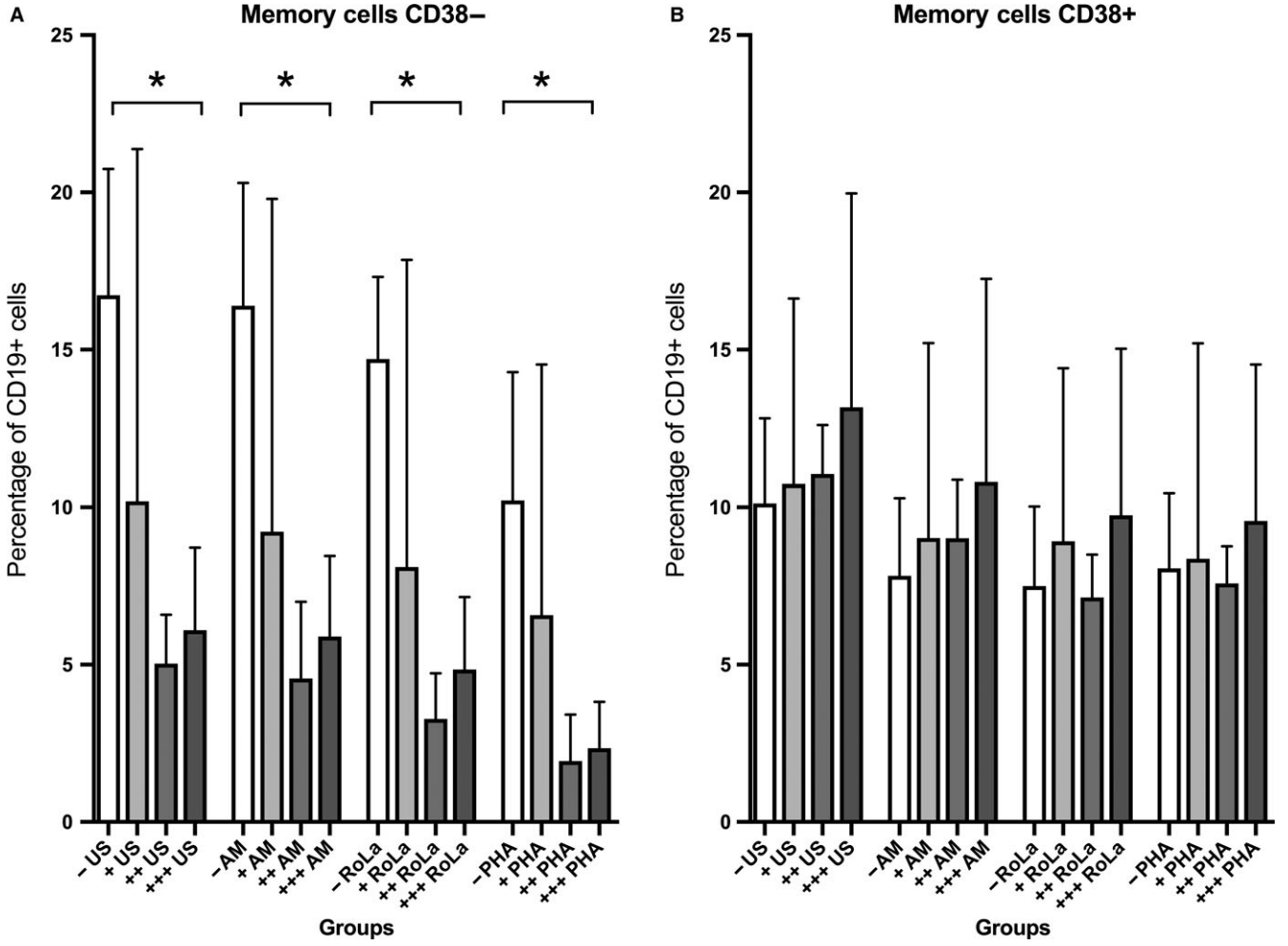

FIGURE 6 $CD38^+$ and $CD38^-$ memory cells. Each column represents the pSS cell samples which are divided according to Ro and La serology (−, +, ++, +++) and culture conditions (US, AM, RoLa, PHA), showing the mean level ± SD. * denotes statistically significant differences ($P < 0.05$).

shown for the RF ≤ 11 and the opposite for the ANA/SSA− group, although not significant (RF ≤ 11 $P = .4258$, ANA/SSA− $P = .1250$). The ESSPRI ≤ 5 group had similar levels of $CD38^-$ MCs and $CD38^+$ MCs.

Considering plasmablasts and plasma cells, the subgroups of RF, ANA/SSA and ESSPRI showed the same pattern—the levels were higher in patients with elevated levels of autoantibodies/highest ESSPRI score. However, the difference was only significant when sorted according to ANA/SSA (PB $P = .0337$, PC $P = .0343$).

4 | DISCUSSION

Sjögren's syndrome (SS) is a chronic autoimmune disease with involvement of a strong B cell component in the etiopathogenesis of the disease. The T follicular helper cells are vital in the formation and maintenance of germinal centres, where they also function as B cell coordinators, selecting promising clones to survive, expand and differentiate.[4] The T_{FH} cells may play an important role when auto-reactive plasma cells are formed in the target tissue of SS patients. In addition, T_{FH} cells may be essential in the lymphoid cell infiltration and formation of germinal centre-like structures in exocrine glands. Sixteen pSS patients and 16 healthy controls were enrolled in the study. Indirect ELISA has been used to analyse plasma samples for Ro52, Ro60 and La48 autoantibodies, whilst PBMCs were used for quantification of distinct subsets by flow cytometry. The focus of this study was to investigate the relation between the T_{FH}-like cells, B cell activity and clinical factors in patients with pSS.

4.1 | Ro52, Ro60 and La48 autoantibody serology

Indirect ELISA was used to examine the presence of Ro52, Ro60 and La48 autoantibodies in plasma samples of

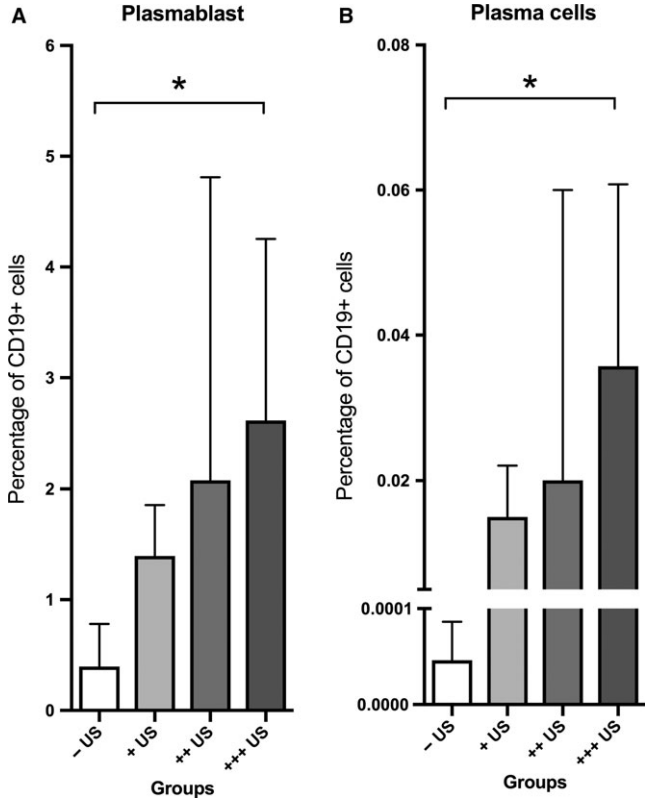

FIGURE 7 Plasmablast and Plasma cell levels in unstimulated samples (US) stratified by Ro52, Ro60, and La48 serology. The patient samples are divided Ro and La serology (– = negative, + = single positive, ++ = double positive, +++ = triple positive). Each bar indicates mean level ±SD. * denotes statistically significant differences ($P < .05$)

healthy controls and pSS patients (Table S3 and Figure S1). We found that 75%, 69% and 50% of the patients expressed Ro52, Ro60 (anti-SSA = 81%) and La48, respectively. The results from in-house ELISA were compared with data from the routine diagnostic lab (Table 1 and Table S2). Only 4 patients showed minor inconsistent serology in the 2 assays, and 2 of them (ie patient no. 8 and 11) had values that were close to cut-off. The clinical data from the Rheumatology Department was obtained when establishing the pSS diagnosis which was not the same time point as the sampling in this study, and thus the serum titres may have drifted between the sample collection time points.[15]

4.2 | Elevated level of ICOS$^+$ T_{FH} -like (ICOS$^+$PD-1$^+$) cells in pSS

The control subjects had a slightly increased number of T helper cells ($CD3^+CD4^+$), but when in addition gating for CXCR5, the difference between the groups was reduced (Figure 1). This indicates that there are less $CD4^+$ T cells in the controls expressing $CXRC5^+$ suggesting less migration against the lymph nodes. We detected a significantly higher level of $ICOS^+PD\text{-}1^+$ ("T_{FH}") cells in pSS patients compared to controls under all tested culture conditions (Figure 2), although the $ICOS^-PD\text{-}1^-$ cells were generally higher in the control group. Since ICOS has a crucial role in B cell differentiation and isotype switching,[6,7] our results suggest that T_{FH}-like cells are to a greater extent activated in pSS patients compared to non-rheumatic individuals. Others have shown that the proportion of circulatory T_{FH} cells correlates with T_{FH} activity in germinal centres.[16,17] Furthermore, several studies have found raised levels of peripheral T_{FH} cells, although defined somewhat differently, in pSS patients compared to controls.[17-19] Interestingly, Szabó et al.[19] reported that the increase in $CD4^+CXCR5^+$ $ICOS^+PD^-1^+$ cells could only be detected in pSS patients with extraglandular manifestations (EGMs). Since clinical data regarding EGM were not obtained in our study, we were not able to investigate this relation in our patient group. In addition, Szabó et al. calculated percentages that were overall higher compared to our findings (eg 0.41 ± 0.28% (EGM+) and 0.23 ± 0.13% (EGM−) vs 0.14 ± 0.12%). Some variance should be expected, as these studies were not identical. While Szabó et al. used fresh samples, we used cryopreserved cells with viability staining and also included the CD3 marker.

4.3 | Lowered number of memory B cells in pSS

We found significantly lower proportions of $CD38^-$ and $CD38^+$ memory B cells in pSS patients compared to controls under all tested conditions (Figure 3), which is consistent with previous reports.[19,20] Memory B cells are resting cells that can quickly differentiate into plasmablasts or plasma cells when activated with specific antigens.[21] Consequently, lowered level of memory B cells in SS patients may indicate previous activation and differentiation into antibody-secreting cells, which already have been suggested by Bohnhorst et al.[22] It has also been postulated that memory B cells accumulate/are retained in inflamed exocrine glands of SS patients, resulting in diminished circulatory memory B cells.[23,24] Both possible explanations suggest that decreased levels of peripheral memory B cells in SS indicate increased B cell activity.

4.4 | A Positive correlation between pSS and the levels of plasmablasts and plasma cells

We observed a significantly higher proportion of plasmablasts in pSS patients compared to controls, showing a positive correlation between plasmablasts and pSS (Figure 3). Previous reports have been somewhat conflicting regarding pSS and circulating plasmablasts/plasma cells. Evaluating pSS patients, Szabó et al. and Jin et al. found

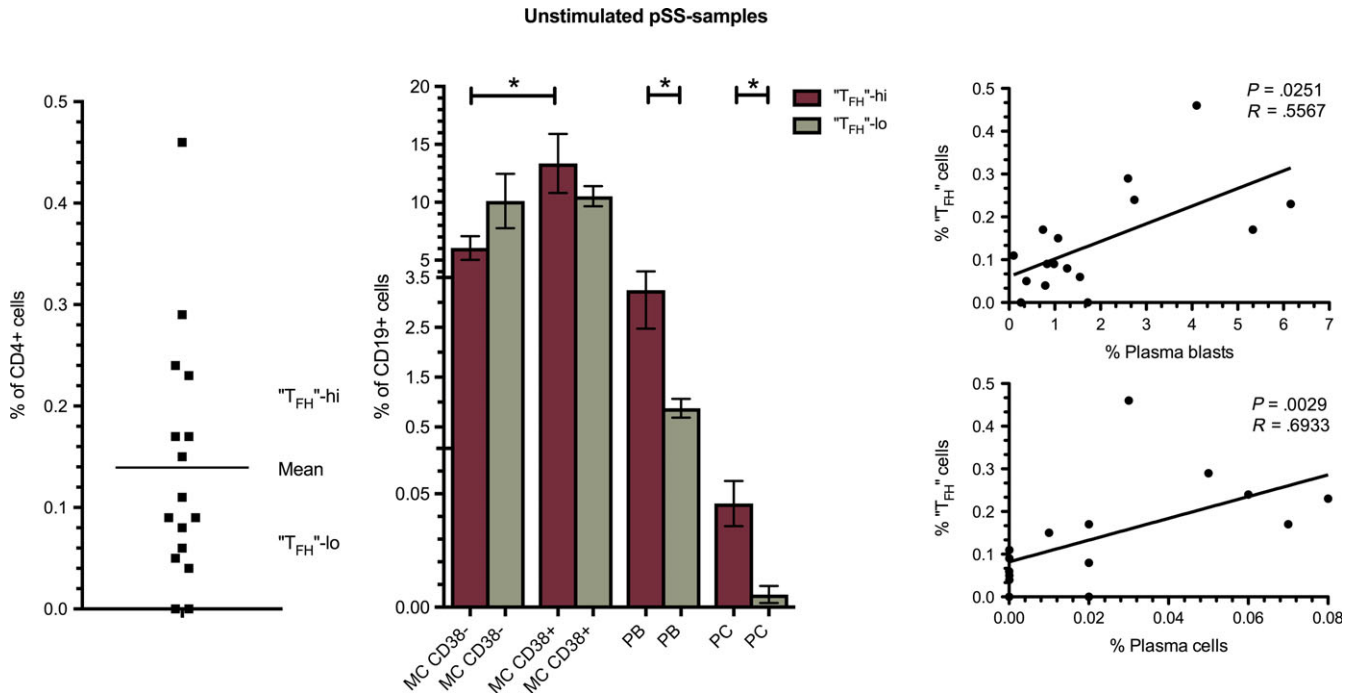

FIGURE 8 Levels of B cell subsets in pSS patients according to the level of "TFH" cells. The graph at the left shows how the "TFH"-profiles were defined. In the middle graph, samples are sorted according to B cell subset and subject group ("TFH"-hi and "TFH"-lo), shown in the legend, and presented as the fraction of CD19 + cells. The lines represent mean ± SEM and significant results ($P < .05$) of interest are marked with an asterisk (*). The graphs at the right are linear correlation plots between "TFH" cells and plasmablasts/plasma cells, shown with a *P*-value (*P*) and Spearman correlation coefficient (R)

decreased numbers of $CD19^{+}CD38^{hi}CD27^{hi}$ plasmablasts and $CD19^{+}CD27^{hi}$ plasma cells, respectively.[19,25] On the contrary, Szyszko et al.[26] found that $CD19^{+}CD38^{+}CD138^{+}$ plasma cells were elevated in pSS patients. The research groups of Szabó and Jin did not stain for the CD138 marker, and therefore plasmablasts cannot be distinguished from plasma cells. However, if we consider our results for plasmablasts and plasma cells as a whole, our findings are inconsistent with Szabó and Jin.

4.5 | Ro/La-specific memory B cells in pSS

We detected significantly lower proportions of memory B cells (both $CD38^{-}$ and $CD38^{+}$ MCs) in patient samples being RoLa-stimulated compared to AM-stimulated (Figure 3). In controls, this association was only made for $CD38^{-}$ MCs. Since all controls were serum negative for Ro/La, this could imply a non-specific response to the Ro/La autoantigens. However, the magnitude of the response differed between the pSS patients and the controls (Figure 3), with the highest decrease observed in patients. The difference was only significant for the $CD38^{-}$ MCs, but the same trend was observed with the $CD38^{+}$ MCs. As discussed earlier, a decreased level of memory B cells may be a sign of activation and differentiation.

4.6 | Stratifying according to Ro/La serology reveal differences in T_{FH} and B cell subsets

When stratifying the pSS patients according to Ro and La serology; negative (−) having no autoantibodies, and single (+), double (++) and triple (+++) positive being seropositive to the Ro52, La60 and La48 autoantigens. We observed a significant increase in the $CXCR5^{+}ICOS^{+}PD\text{-}1^{+}$ cell population in the triple positive compared to the seronegative (Figure 5), but not in the other $CXCR5^{+}$ subpopulations. When continuing the stratification on the memory cell populations ($CD27^{+}$), a significant decrease in the $CD38^{-}$ cells (Figure 6) was observed. In addition, an increase in both plasmablasts and plasma cells (Figure 7) in the triple positive patients was noted. Regarding the "T_{FH}" cells, our findings are consistent with Szabó et al.,[27] who found a positive correlation between serum levels of SSA and percentage of circulating T_{FH} cells.

4.7 | Level of "T_{FH}" cells correlates with levels of plasmablasts and plasma cells in pSS

We defined 2 groups of pSS patients based on their levels of "T_{FH}" cells (Figure 8). Those categorized as "T_{FH}-hi," expressed significantly higher levels of plasmablasts and

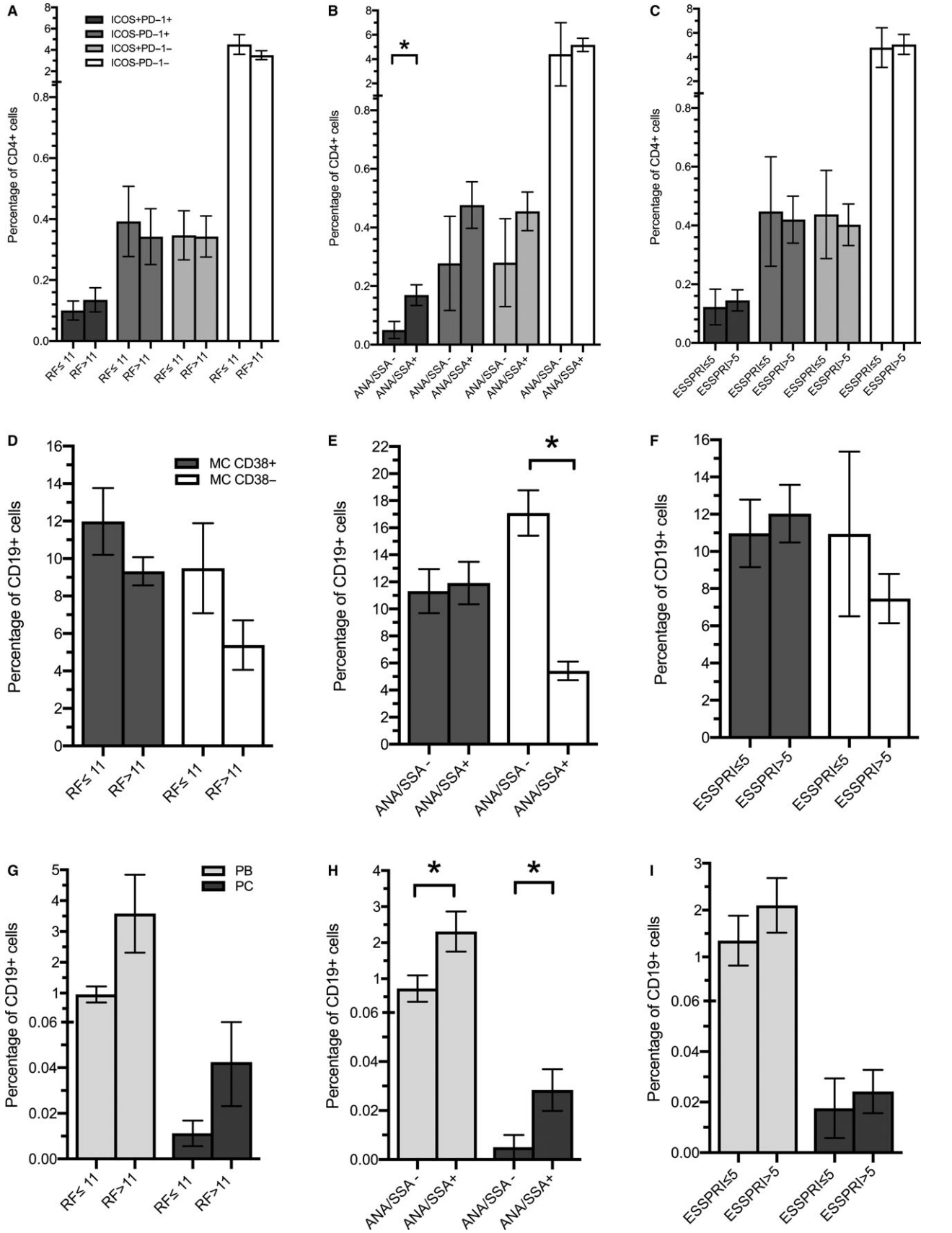

FIGURE 9 Levels of T_{FH}-like cells and B cell subsets in pSS patients grouped according to clinical data. The samples were sorted according to subject group (i.e. RF, ANA/SSA and ESSDAI) shown in legend, and presented as the fraction of $CD4^+/CD19^+$ cells. The lines represent mean ± SEM and significant results ($P < 0.05$) of interest are marked with an asterisk (*).

plasma cells compared to subjects in the "T_{FH}-lo" group. The level of "T_{FH}" cells was also shown to correlate positively with the levels of plasmablasts and plasma cells. In addition, the "T_{FH}-hi" group had a significantly higher level of $CD38^+$ memory B cells compared to $CD38^-$ memory B cells. In the "T_{FH}-lo" group, these levels were similar. The observed skewing towards $CD38^+$ MCs in "T_{FH}-hi" patients is therefore suggestive of a connection between "T_{FH}" cells and activated memory B cells. Overall, we believe these findings substantiate the role of the "T_{FH} cells" in B cell activity.

4.8 | Association between investigated cell subsets and disease activity in pSS

We divided the pSS patients into groups according to clinical features (ie FS, RF, ANA/SSA and ESSPRI). Although a significant association between focus score (FS) and other disease activity parameters have been found in other pSS cohorts previously, only trends could be noted in our patient group. One explanation for this could be that FS were generally low in our patient groups (Table 1). Even though, a general pattern of increased levels of T_{FH}-like cells was observed in ANA/SSA+ patients compared to ANA/SSA− patients (only significant for $ICOS^+PD\text{-}1^+$ cells; Figure 9). This association could not be made when grouped according to RF and ESSPRI. We found a trend of reduced $CD38^-$ memory B cells levels in patients with indications of increased disease activity (ie ANA/SSA+, RF > 11 and ESSPRI > 5), (Figure 9), although only significant for the ANA/SSA subdivision. On the other hand, the $CD38^+$ MCs were more stable according to disease activity parameters. In contrast, the plasma cells and blast levels were higher in the groups with higher disease activity score. We may not have got strong statistical evidence, but taken together, our observations indicate a trend that the disease activity also skews the general PBMC population subsets, to higher T_{FH}, lower memory B cells, and more activated B cells.

5 | CONCLUSION

IL-21 is considered to be a signature molecule of T_{FH} cells, which has been found elevated in several autoimmune disorders including pSS.[28,29] However, other cells may also produce IL-21.[30,31] Verstappen et al.[32] showed a relation between T_{FH} and B cells, showing that T_{FH} cells were reduced in pSS patients undergoing B cell depletion therapy, and this reduction was associated with improvement of objective disease activity parameters (eg ESSDAI, IgG, SWS). IL-6 (in addition to IL-21) is thought to be important in the differentiation pathway of T_{FH} cells[4] and Pollard et al[33] have previously reported that B-cell depletion resulted in decreased serum concentration of IL-6 in pSS patients. Based on this, Verstappen et al postulated that the observed decrease in circulating T_{FH} cells might be the result of depletion of IL-6 producing B cells. They suggest there is a crosstalk between B cells and $CD4^+$ T cells, which could be a promising target for pSS therapy.[32]

In conclusion, we found that the pSS patients had lowered levels of memory B cells and raised level of $ICOS^+$ T_{FH}-like cells and plasmablasts. The proportion of T_{FH} ($ICOS^+PD\text{-}1^+$) cells was positively correlated with the proportion of plasmablasts and plasma cells and are in concordance with earlier reports.[17-19] The level of T_{FH} could have implication on the level of Ro and La specific memory B cells and on Ro and La serology due to the positive correlation between these factors. We were not able to observe a link between T_{FH} levels and disease activity, but there was a correlation between T_{FH} levels ($ICOS^+PD\text{-}1^+$) and ANA+. Overall, we believe our findings indicate an association between the T_{FH}-like cells and B cell activity in pSS, which may have an implication in the disease development, but further studies have to be carried out to elucidate this.

ORCID

K. A. Brokstad http://orcid.org/0000-0003-0593-2731

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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