

SHORT REPORT

Association between T follicular helper cells and T peripheral helper cells with B-cell biomarkers and disease activity in primary Sjögren syndrome

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INTRODUCTION

Primary Sjögren syndrome (pSS) is a systemic autoimmune disease, characterised by an infiltration of the exocrine glands, leading to sicca syndrome. Extraglandular systemic involvements (such as pulmonary, renal or neurological involvement) occur in about 30% of the patients. One of the most severe complications is the development of B cell lymphoma.¹ pSS is characterised by chronic B cell activation, as evidenced by the presence of anti-SSA (Sjögren syndrome A antigen), anti-SSB (Sjögren syndrome B antigen) antibodies, rheumatoid factor (RF) and hypergammaglobulinaemia.² Using mass cytometry (CyTOF), we have shown an expansion of plasma cells in minor salivary gland and an expansion of circulating plasmablasts in pSS patients, compared with controls.³ Ectopic germinal centres (EGC) in minor salivary glands are present in about one-quarter of the patients and could enhance local production of autoantibodies. T cells also participate to the pathophysiology of pSS, as suggested by the presence of a T lymphocyte infiltrate within the patients' minor salivary gland biopsies,⁴ as well as the association of pSS with several HLA class II alleles.⁵ In addition, there is an association between pSS and a single-nucleotide polymorphism located within *CXCR5* locus that codes for a key chemokine expressed by T follicular helper (Tfh) cells.

Tfh are a subset of T lymphocytes specialised in providing help to B cells, and are essential for EGC formation, recruitment (via CXCL13) and activation (via ICOS) of B cells, affinity maturation and development of memory B cells and plasmablasts (via secretion of interleukin 21 (IL-21)). They are CXCR5⁺, PD-1⁺ and ICOS⁺, the latter being

Key messages

What is already known about this subject?

- T follicular helper cells (Tfh) are elevated in the blood of patients with primary Sjögren syndrome (pSS) and activated Tfh correlate with disease activity.

What does this study add?

- T peripheral helper (Tph) cells are a newly described subtype of T lymphocytes. They are elevated in the blood of patients with pSS and correlate with circulating plasmablasts.

How might this impact on clinical practice?

- As Tfh and Tph might promote B-cells activity in pSS, they could be a potential therapeutic target in this disease.

considered as an activation marker.⁶ Several studies supported their involvement in pSS: (1) increased levels of Tfh in blood and within minor salivary glands in pSS compared with controls,^{7–9} (2) correlation between activated Tfh and disease activity (ESSDAI (EULAR Sjögren's syndrome disease activity index),^{9 10} clinESSDAI¹⁰), (3) correlation between IL-21 and disease activity and autoantibodies status^{7 11 12} and (4) ability of the epithelial cells to induce Tfh.¹² Another T cell subset has been recently described in inflamed tissues such as joint, but also in the blood of patients with rheumatoid arthritis¹³: the T peripheral helper cells (Tph) that are characterised by CD4⁺PD1^{hi}CXCR5⁺. In contrast to Tfh, these cells lack CXCR5 but similar to Tfh, they express IL-21, CXCL13, ICOS and induce plasmablasts differentiation *in vitro*.¹³ In SLE, their expansion correlates with disease activity.^{14 15} Two studies have shown that circulating Tph were higher in patients with pSS compared with controls.^{8 16}

Given the role of B cell activation in pSS, we thought it would be relevant to study these cells' subtypes in pSS.

Thus, in this study, we aimed to assess Tfh and Tph in pSS, to look at their activation profile and their association with disease characteristics.

PATIENTS AND METHODS

Patients and controls

Patients were referred to the Department of Rheumatology of Hôpitaux Universitaires Paris-Sud (France), a tertiary centre, reference centre for systemic autoimmune diseases. The inclusion criteria were: age >18 years, diagnosis of pSS according to the American European Consensus criteria or the 2016 ACR (American College of Rheumatology) /EULAR criteria. Patients who received an anti-CD20 therapy or any immunosuppressive drug in the last 6 months or were treated with steroids >10 mg of prednisone per day were excluded. Clinical (sex, age, treatment, ESSDAI, lymphoma, cryoglobulinemic vasculitis), biological (anti-SSA or SSB antibodies, gammaglobulins level, RF) and pathological (result of the minor salivary gland biopsy) characteristics were available in the patients' medical file. Hypergammaglobulinaemia was defined as a level of gammaglobulins higher than 12.5 g/L.

Two different cohorts were used. Patients included in the cohort I were assessed using CyTOF based on a previous study performed by our group.³ Control subjects were either blood donors (from the French Blood Establishment) or patients with sicca syndrome without any pSS or other immune or infectious disease. Clinical evaluation confirmed that the latter had neither autoantibodies nor a positive biopsy result. To validate the first findings obtained with cohort I, we used an independent cohort of replication (cohort II) with assessment of Tph and Tfh by flow CyTOF and adding ICOS as a marker of activation. Controls in the cohort II were exclusively blood donors. Informed consent was obtained from all patients and controls. Blood samples were collected in heparinised tubes.

Mass CyTOF analysis

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient and then frozen stored at -80°C as previously described.³ Samples were stained and prepared for CyTOF analysis, by using an optimised cocktail of 34 metal-conjugated antibodies designed to identify 40 major and minor human blood cell subsets. After acquisition on a CyTOF II (Fluidigm, South San Francisco, California, USA), data were normalised to internal bead standards. Tfh were defined as $\text{CD4}^+\text{CXCR5}^+\text{PD1}^+$, Tph as $\text{CD4}^+\text{CXCR5}^+\text{PD1}^{\text{hi}}$, plasmablasts as $\text{CD19}^+\text{CD27}^+\text{CD38}^{\text{hi}}$.

Flow CyTOF analysis

PBMCs were isolated by Ficoll gradient and freshly stained. Cells were analysed on a BD FACSCanto II flow cytometer. Data were analysed using Flow Jo, version 10.

Tfh were defined as $\text{CD4}^+\text{CXCR5}^+\text{PD1}^+$, Tph as $\text{CD4}^+\text{CXCR5}^+\text{PD1}^{\text{hi}}$, activated Tfh as $\text{CD4}^+\text{CXCR5}^+\text{PD1}^+\text{ICOS}^+$, activated Tph as $\text{CD4}^+\text{CXCR5}^+\text{PD1}^{\text{hi}}\text{ICOS}^+$. Plasmablasts were defined as $\text{CD19}^+\text{CD27}^+\text{CD38}^{\text{hi}}$. Unfortunately, we encountered a technical issue with the CD38 staining (high amount of false positive staining) and thus plasmablasts could not be analysed by flow CyTOF. Detail of fluorochrome-conjugated antibodies used is provided in supplementary. Gating strategy is presented in online supplemental figure S1.

Statistical analysis

Data were expressed as median (IQR). Comparisons between groups were performed using Mann-Whitney U or Kruskal-Wallis test. Correlations were analysed using Spearman's rank correlation coefficients. All analyses were performed using GraphPad Prism V.7.02 (GraphPad Software, San Diego, California, USA). A value of $p < 0.05$ was considered as statistically significant.

RESULTS

Patients' characteristics

We included 29 patients and 23 healthy controls (HC) for mass CyTOF analysis (cohort I). A second cohort including 15 patients and 19 HC was used for flow CyTOF analysis (cohort II). Controls in cohort I were mostly women ($n=21$, 91.3%) and their median age (IQR) was 54 (29) years. Controls in cohort II were mostly women ($n=15$, 79.0%) and their median age (IQR) was 39 (14.5) years. Patients' characteristics are presented in table 1.

Expansion of Tfh and Tph in blood of pSS patients

We analysed circulating Tfh ($\text{CD4}^+\text{CXCR5}^+\text{PD1}^+$) and Tph ($\text{CD4}^+\text{CXCR5}^+\text{PD1}^{\text{hi}}$) in cohort I using mass CyTOF. The median (IQR) percentage of Tfh among CD4^+ T cells was significantly higher in pSS patients compared with controls: 5.9 (4.8) % vs 2.9 (1.9) % ($p < 0.001$) (figure 1A), as well as the median percentage of Tph among CD4^+ T cells: 4.7 (4.3) % vs 3.2 (3.2) % ($p < 0.01$) (figure 1B). We confirmed these results in cohort II using flow CyTOF. The median percentage of circulating Tfh and circulating Tph was increased in pSS patients compared with HC, respectively: 9.7 (7.2) % vs 4.1 (4.8) % ($p < 0.001$) for Tfh (figure 1C) and 4.5 (3.1) % vs 1.8 (4.1) % ($p < 0.01$) for Tph (figure 1D).

Then, we evaluated in cohort II the activation status of these subsets, by defining activated Tfh as $\text{CD4}^+\text{CXCR5}^+\text{PD1}^+\text{ICOS}^+$ and activated Tph as $\text{CD4}^+\text{CXCR5}^+\text{PD1}^{\text{hi}}\text{ICOS}^+$. Activated Tfh were increased in patients with pSS compared with HC: 3.9 (2.4) % vs 1.2 (1.7) % ($p < 0.01$) (figure 1E), as well as activated Tph: 1.0 (1.7) % vs 0.4 (0.5) % ($p < 0.001$) (figure 1F).

Association of Tfh and Tph with disease activity

We next studied whether these subsets were associated with disease activity assessed by ESSDAI in flow CyTOF. We found a positive moderate correlation between ESSDAI and the percentage of Tfh ($r=0.59$, $p < 0.05$)

Table 1 Patients' characteristics

	Cohort I (n=29)	Cohort II (n=15)
Sex (female)	29 (100%)	15 (100%)
Age (median±IQR), years	54.0±23	64±28
ESSDAI score (median, ranges)	2 (0–40)	4 (0–17)
Systemic manifestations		
Pulmonary involvement	0 (0%)	3 (20%)
Cutaneous involvement	2 (6.0%)	2 (13.3%)
Swelling of parotid glands	5 (17.2%)	2 (13.3%)
Lymphoma	NA	1 (6.7%)
Others	3 (10.3%)	1 (6.7%)
Anti-SSA antibody	20/28 (71.4%)	11/15 (73.3%)
Anti-SSB antibody	10/28 (35.7%)	5/14 (35.7%)
Rheumatoid factor	9/17 (52.9%)	7/15 (46.7%)
Gammaglobulins levels (mean±SD)	15.3±6.3 g/L	14.0±4.4 g/L
Lymphocytes (mean±SD)	1.4±0.6×10 ⁹ /L	1.7±0.7×10 ⁹ /L
Biological cryoglobulinaemia	NA	1 (6.7%)
Current treatment	–	–
Corticosteroids <10 mg/day	1 (3.5%)	1 (6.7%)
Hydroxychloroquine	9 (31%)	1 (6.7%)
Rituximab (>6 months before)	1 (3.5%)	4 (26.6%)
Belimumab (>6 months before)	1 (3.5%)	0 (0%)

NA, not available.

(figure 2A), and between ESSDAI and the percentage of activated Tfh (ICOS⁺) ($r=0.58$, $p<0.05$) (figure 2B). Conversely, no significant correlation was found between the percentage of Tph and ESSDAI ($r=0.21$, $p=0.45$) nor between the percentage of activated Tph and ESSDAI ($r=0.28$, $p=0.33$) (online supplemental figure S2, A and B). There was no correlation between clinESSDAI and Tfh, activated Tfh, Tph or activated Tph (online supplemental figure S2, C, D, E and F).

Association of Tfh and Tph with B cell activation markers

Then, we looked for an association between Tfh, Tph, activated Tfh, activated Tph and B-cell markers including plasmablasts (defined as CD19⁺CD27⁺CD38^{hi}), hypergammaglobulinaemia (defined as gammaglobulins >12.5 g/L), positivity for anti-SSA and RF. Using CyTOF, we found a positive moderate correlation between the percentage of plasmablasts and the percentage of Tfh ($r=0.43$, $p<0.01$) (figure 2C) and Tph ($r=0.53$, $p<0.0001$) (figure 2D), respectively. Plasmablasts characterisation was impossible in flow CyTOF for technical issues.

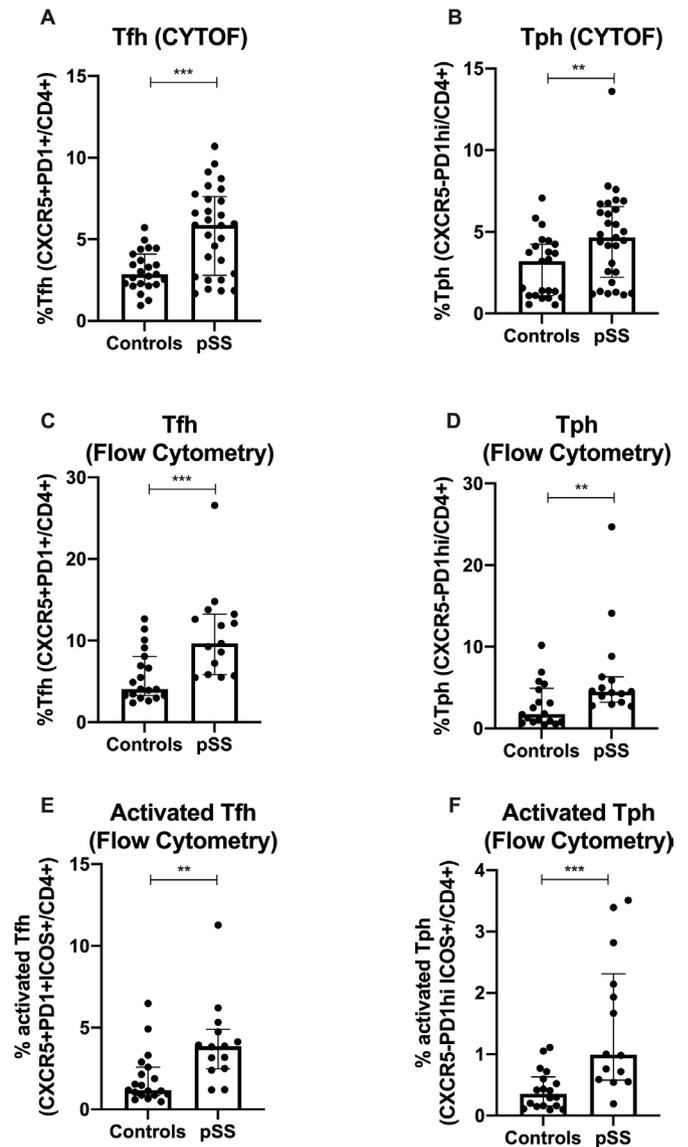


Figure 1 Tfh and Tph are expanded in the blood of patients with primary Sjögren syndrome (pSS). (A) Median percentage of Tfh among CD4⁺ in the blood of patients with pSS (n=29) and in healthy controls (HC) (n=23) in mass cytometry (CyTOF). (B) Median percentage of Tph among CD4⁺ in the blood of patients with PSS (n=29) and in HC (n=23) in mass CyTOF. (C) Median percentage of Tfh among CD4⁺ in the blood of patients with pSS (n=15) and in HC (n=19) in flow CyTOF. (D) Median percentage of Tph among CD4⁺ in the blood of patients with PSS (n=15) and in HC (n=18) in flow CyTOF. (E) Median percentage of activated Tfh among CD4⁺ in the blood of patients with PSS (n=14) and in HC (n=19) in flow CyTOF. (F) Median percentage of activated Tph among CD4⁺ in the blood of patients with pSS (n=14) and in HC (n=18) in flow CyTOF. Data represent the median with IQR; **p<0.01, ***p<0.001 by Mann-Whitney U test. Tfh, T follicular helper; Tph, T peripheral helper.

In flow CyTOF, median percentage of activated Tfh were significantly higher in patients with hypergammaglobulinaemia, compared with patients without hypergammaglobulinemia: 4.0 (1.9) % vs 2.5 (1.5) % ($p<0.05$) (figure 2E). Using a threshold of 16 g/L to define

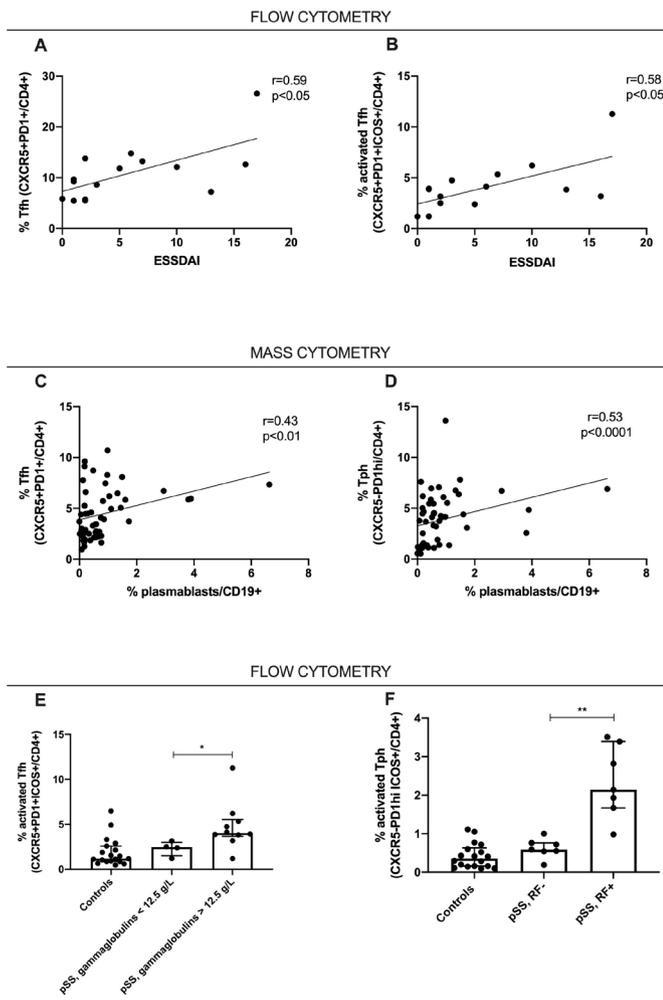


Figure 2 Correlation between Tfh, Tph and disease activity and B cells biomarkers. Correlations between ESSDAI score and circulating Tfh (A) and activated Tfh (B) assessed in flow cytometry in patients with PSS. Correlations between the percentages of plasmablasts and Tfh (C) and Tph (D) assessed in mass cytometry. Circulating activated Tfh according to gamma globulins level (E) assessed in flow cytometry. circulating activated Tph according to rheumatoid factor status (F) assessed in flow cytometry. data represent the median (IQR); * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ by Mann-Whitney U test. Correlations were made using Spearman test. pSS, primary Sjögren's syndrome; Tfh, T follicular helper; Tph, T peripheral helper.

hypergammaglobulinemia, as used in ESSDAI, activated Tfh were still increased in pSS patients with hypergammaglobulinemia compared with patients without (4.6% vs 2.8% ($p=0.0593$)). Activated Tfh did not differ between patients with or without RF (3.9% vs 3.2%) (online supplemental figure S3F), nor between patients with or without anti-SSA (3.8% vs 4.1%) (online supplemental figure S3E) or SSB antibodies (3.8% vs 3.6%) (online supplemental figure S3H). Median percentage of activated Tph were significantly higher in patients with RF compared with patients without RF: 2.1 (1.7) % vs 0.6 (0.2) % ($p<0.01$) (figure 2F) and in patients with anti-SSB antibodies compared with those without anti-SSB antibodies: 2.8 (2.1) % vs 0.7 (0.5) % ($p<0.05$)

(online supplemental figure S3P). Activated Tph did not differ between patients with or without hypergammaglobulinaemia (1.3% vs 0.7%) (online supplemental figure S3O), nor between patients with or without anti-SSA antibodies (1.0% vs 0.6%) (online supplemental figure S3M).

DISCUSSION

Our study demonstrates that Tfh and Tph, as well as activated Tfh and Tph are expanded in pSS patients. It shows a correlation between Tfh and activated Tfh and disease activity, and an association between activated Tph and Tfh and some B-cell markers.

Our results regarding activated Tfh in pSS are consistent with previous studies that have shown an expansion of circulating activated Tfh in patients with pSS,^{16,17} mostly in those with extraglandular manifestations and/or high disease activity^{7,8} and a correlation between activated Tfh and disease activity.^{9,10} On the opposite, in another study, pSS patients had a normal frequency of blood activated Tfh, but an increased level of T follicular regulatory (Tfr) cells and blood Tfr/Tfh ratio.⁹

In addition, our study focuses on Tph in pSS. Indeed, Tph have now been studied in many auto-immune diseases, such as systemic erythematosus lupus, systemic sclerosis or rheumatoid arthritis,¹⁸⁻²⁰ but only few data are available in pSS.^{16,17} We confirmed their expansion in the blood of pSS patients and our study is one of the first to describe their association with clinical and biological characteristics.

Our results might suggest a potential role of activated Tph and Tfh in activation and differentiation of B-cells, as suggested by the association between these cells and some B cells markers (gammaglobulins, RF, anti-SSB antibodies). Regarding these hypothesis, Verstappen *et al* have shown that Abatacept was able to reduce the percentage and the number of circulating Tfh in pSS patients and, interestingly, this reduction contributes to attenuated B cell biomarkers such as circulating plasmablasts, anti-SSA and anti-SSB.⁸

One limitation of our study is the definition of Tph, which probably lacks of specificity, as this subset defined as CD4⁺CXCR5⁺PD1^{hi} could include other types of activated helper CD4 or pre-Tfh (as Tfh express PD1 before expressing CXCR5 (6)). To limit this bias, we have focused on the PD1^{hi} subset and not only on the whole PD1⁺ subset. Regarding Tfh, we defined them as CD4⁺CXCR5⁺PD1⁺ as previously described.⁷ However, in other studies,^{9,10} CD25, FOXP3 and CD45RO/RA are used to define this population and distinguish them from T follicular regulatory cells (Tfr). Thus, we cannot exclude a contamination of our defined Tfh population by Tfr that is nevertheless a minute population.

Our study focused on circulating Tfh and Tph but did not explore those cells in the salivary glands. It is interesting to note that PD1⁺ICOS⁺T cells were found at an increased frequency in pSS salivary glands compared with controls.⁹ A recently published article from Pontarini *et al* described that both Tfh and Tph-cells were enriched in salivary glands with germinal centres from pSS patients.¹⁶

Another limitation was the age difference between controls and patients in the cohort II. Tfh have been shown to increase with ageing.²¹ However, age in patients and controls was similar in the cohort I and this difference do not impact results focused on pSS (correlations with disease's parameters).

What's more, it can be noted that, in our study, the proportion of Tfh and Tph detected by CyTOF and flow CyTOF differ. This is due to the differences between these two techniques, and to a weak staining with CXCR5 and PD1 in CyTOF. However, we were able to detect the same difference between patients and controls using these two techniques which supports the validity of our results.

In conclusion, our study demonstrates that Tfh and Tph are increased in the blood of patients with pSS and might promote B cells activation. Thus, they could be potential new therapeutic targets in this disease, by inhibiting the crosstalk between these cells and B cells.

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Competing interests MM is employed by BIOGEN.

Patient consent for publication Not required.

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