T cell-dependent B cell hyperactivity in primary Sjögren's syndrome

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T FOLLICULAR REGULATORY CELLS FROM PATIENTS WITH PRIMARY SJÖGREN'S SYNDROME EXPRESS DECREASED LEVELS OF CTLA-4

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Work in progress

ABSTRACT

Objective

Humoral immune responses rely to a large extent on T follicular helper (Tfh) cells. T follicular regulatory (Tfr) cells are important regulators of Tfh cells. Proportions of circulating Tfh (cTfh) cells are increased in primary Sjögren's syndrome (pSS) patients compared with healthy controls (HCs). Tfh cells probably play a critical role in the enhanced activation of B cells in pSS. This study aimed to assess the frequency and phenotype of cTfh cells and circulating Tfr (cTfr) cells in pSS, as well as their relation to B cell activity and systemic disease activity.

Methods

Sixty-eight pSS patients (66 female, mean age 50) classified according to the ACR-EULAR criteria and 24 HCs (23 female, mean age 43) were included. Cryopreserved peripheral blood mononuclear cells were analyzed by flow cytometry to assess the frequency of cTfh cells ($CD45RA^-FoxP3^-CXCR5^+PD\text{-}1^+$) and cTfr cells ($CD45RA^-FoxP3^+CXCR5^+PD\text{-}1^+$). Median expression levels of CTLA-4 per cell were also measured.

Results

Frequencies of both cTfh cells and cTfr cells were elevated in pSS patients compared with HCs ($P<0.001$). Circulating Tfr cells were even more increased than cTfh cells, resulting in significantly higher cTfr/cTfh ratios in pSS ($P<0.001$). Frequencies of cTfh and cTfr cells correlated positively with serum levels of IgG and CXCL13, and systemic disease activity. In pSS patients, expression levels of CTLA-4 in cTfr cells were significantly decreased compared with HCs ($P<0.001$). Lower expression levels of CTLA-4 in cTfr cells tended to be associated with an increase in cTfh cells ($\rho=-0.382$, $P=0.07$).

Conclusion

The cTfr/cTfh ratio is increased in pSS patients and frequencies of both subsets correlate with measures of B cell activity and systemic disease activity. Circulating Tfr cells from pSS patients express lower levels of CTLA-4, suggesting that despite their increased frequencies, their ability to suppress is impaired. A reduced suppressive capacity of Tfr cells may contribute to the expansion of Tfh cells and consequently to B cell hyperactivity in pSS.

INTRODUCTION

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease characterized by lymphocytic infiltration of salivary and lacrimal glands. B cell hyperactivity is a key feature of pSS pathogenesis, and is, among others, reflected by elevated serum IgG levels [1]. T follicular helper (Tfh) cells, an effector subset of $CD4^+$ T cells, probably play a critical role in the enhanced activation of B cells. An important mechanism of B cell help by Tfh cells is the production of IL-21. This cytokine promotes B cell proliferation and differentiation towards plasma cells [2]. Costimulation and IL-21 production by Tfh cells are also crucial for somatic hypermutation, affinity maturation and class switch recombination of B cells within germinal centers (GC) [3].

Tfh cell-mediated B cell help is naturally regulated by T follicular regulatory (Tfr) cells. Tfr cells are a specialized subset of regulatory T (Treg) cells and are able to suppress both Tfh cells and B cells within the GC [4–6]. In particular the ratio between Tfh cells and Tfr cells seems to control the magnitude of the antibody response [7]. An important mode of suppression by Treg cells is through the expression of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). This inhibitory receptor is even more highly expressed on Tfr cells [5,8,9]. Possible mechanisms of suppression by Treg cells through CTLA-4 are I) physical sequestering of CD80/86 on antigen-presenting cells (APC), hereby attenuating CD28 signaling and T cell activation, II) transendocytosis of CD80/86 ligands upon interaction with APCs, and III) intrinsic competition with CD28 for CD80/86 binding at the immune synapse [10].

While several studies have shown that proportions of circulating Tfh (cTfh) cells are increased in pSS patients compared with healthy controls (HCs) [11–15], the prevalence of cTfr cells in pSS is rather unexplored. Circulating cTfh and cTfr cells are thought to function as a memory pool to enable fast and strong, but controlled responses upon antigen re-encounter, although the suppressive capacity of circulating Tfr (cTfr) cells seems lower compared to Tfr cells in the GC [16]. Changes in proportions of Tfr cells and expression of inhibitory receptors, such as CTLA-4, may affect Tfh cell formation and function, as well as B cell responses. The aim of this study was to assess the frequency and phenotype of cTfr cells and cTfh cells in pSS. Furthermore, the relation between cTfr and cTfh cell frequencies, B cell activity, and systemic disease activity were studied. We found that cTfr cells were expanded in pSS patients, even to a larger extent than cTfh cells. The frequency of cTfr cells correlated positively with serum levels of IgG and CXCL13, and with systemic disease activity. Finally, we observed that circulating Treg cells, and in particular cTfr cells, from pSS patients express decreased levels of CTLA-4.

PATIENTS AND METHODS

Study population

Twenty-four pSS patients (23 female, mean age 44 years) and 24 sex- and age-matched HCs (23 female, mean age 43 years) were included. All pSS patients participated in a previously reported, open-label re-treatment study with rituximab [17]. Twenty-four patients of this cohort were analyzed in the current study; 4 patients were not included because of unavailability of stored peripheral blood mononuclear cell (PBMC) samples (n=3) or serum sickness-like manifestations after the first dose of RTX (n=1). Patients were included at least one year after the last rituximab infusion, after reappearance of circulating B cells to baseline levels, and recurrence of symptoms. Only baseline samples before start of re-treatment were included in the current study. Characteristics of the study population have been described previously [14,18]. All patients retrospectively fulfilled 2016 ACR-EULAR criteria for pSS [19]. In addition, samples of 44 recently diagnosed, biologic treatment-naive pSS patients, included in an inception cohort, were analyzed (43 female, mean age 53 years). These patients prospectively fulfilled 2016 ACR-EULAR criteria for pSS.

For all patients included in the current study, systemic disease activity was prospectively assessed by the ESSDAI and Clinical ESSDAI (ESSDAI without inclusion of the biological domain) [20,21]. All patients and HCs provided written informed consent. The study was approved by the Medical Ethics Committee of the University Medical Center Groningen (METc2008.179/METc2013.066).

Flow cytometry analysis

Cryopreserved PBMC samples were thawed and of each sample two million cells were fluorescently labeled for immunophenotyping of $CD4^+$ T cells by flow cytometry. A previously reported panel of antibodies was used [14], with addition of anti-human CTLA-4-BV421 (clone BNI3, BD Biosciences) for the 24 baseline samples of the rituximab cohort and HCs. Both membrane-bound and intracellular CTLA-4 expression was measured, because it is highly endocytic, resulting in approximately 90% of CTLA-4 being intracellular [10]. The Foxp3 transcription factor fixation/permeabilization concentrate and diluent solutions (eBioscience) were used for staining of CTLA-4 (and FoxP3).

Circulating Tfh cells were defined as $CD4^+CD45RA^-FoxP3^-CXCR5^+PD\text{-}1^+$, cTfr cells as $CD4^+CD45RA^-FoxP3^+CXCR5^+PD\text{-}1^+$ and peripheral Treg (pTreg) cells as $CD4^+CD45RA^-FoxP3^+CXCR5^-$ cells. Median expression levels per cell of CTLA-4 in cTfr cells and pTreg cells were measured by median fluorescence intensity (MFI). Flow cytometric measurements were performed on a FACS-LSRII flow cytometer (Becton Dickinson) and data were analyzed using FlowJo software (Tree Star).

Analysis of CXCL13 levels in serum

In patients from the inception cohort, serum levels of CXCL13 were measured by an enzyme-linked immunosorbent assay (ELISA) using the DuoSet ELISA Human CXCL13 development system (R&D systems), according to the manufacturer's protocol.

Statistical analysis

Statistical analyses for comparisons between pSS patients and HCs were performed using Mann–Whitney U test. Correlations were evaluated with Spearman's Rho tests. Two-tailed P values <0.05 were considered statistically significant.

RESULTS

Frequency and phenotype of cTfh cells and cTfr cells in pSS

Immunophenotyping of $CD4^+$ T cells revealed that pSS patients presented with higher frequencies of circulating cTfh cells, as well as cTfr cells, compared with HCs (Figure 1A and 1B; P<0.001). Circulating Tfr cells were relatively further increased than cTfh cells, resulting in significantly higher cTfr/cTfh ratios in pSS patients, compared with HCs (Figure 1C; P<0.001). Frequencies of cTfh cells and cTfr cells were comparable between pSS patients from the rituximab cohort and the inception cohort, indicating that treatment with rituximab in the past had no effect on these frequencies.

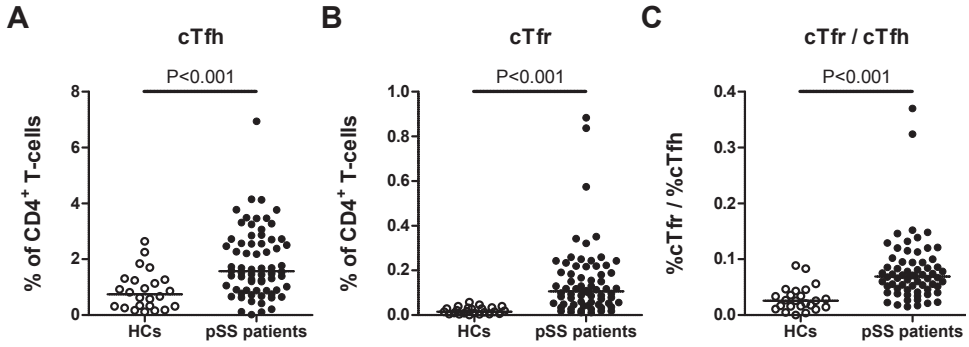

FIGURE 1 | Frequencies of cTfh cells, cTfr cells and the cTfr/cTfh ratio in pSS patients and healthy controls. Within the $CD4^+$ T-cell compartment, frequencies of (A) cTfh cells ($CD45RA^-FoxP3^-CXCR5^+PD\text{-}1^+$) and (B) cTfr cells ($CD4^+CD45RA^-FoxP3^+CXCR5^+PD\text{-}1^+$) were assessed and the cTfr/cTfh ratio (C) was calculated. Baseline data from pSS patients in the rituximab group (n=24) and data from the inception cohort (n=44) were combined. HC: healthy control. Horizontal lines indicate the median. P-values were calculated using the nonparametric Mann-Whitney U-test.

To investigate the migratory potential of circulating cTfh cells and cTfr cells to inflamed tissue in pSS, expression of CXCR3 and CCR6 was measured. We found larger proportions of CXCR3-expressing cTfh and cTfr cells in pSS patients compared with HCs (Figure 2). The $CXCR3^{+}CCR6^{-}$ phenotype, similar to Th1 cells [22], was predominant in pSS, both for cTfh cells (median 67%) and cTfr cells (median 44%). Frequencies of cTfh and cTfr cells with a $CXCR3^{-}CCR6^{+}$ phenotype, similar to Th17 cells, were not significantly different between pSS patients and HCs (Figure 2).

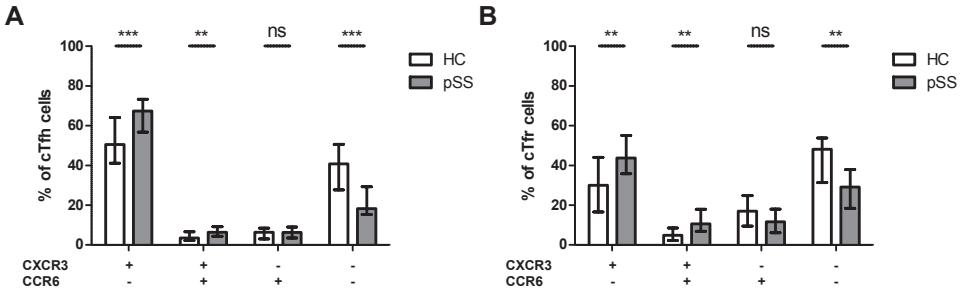

FIGURE 2 | Chemokine receptor expression by cTfh and cTfr cells. Expression of CXCR3 and CCR6 by circulating T follicular helper (cTfh) cells (A) and circulating T follicular regulatory (cTfr) cells (B) was measured in 24 pSS patients and 24 age- and sex-matched HCs. Bars indicate the median and interquartile range. P-values were calculated using the nonparametric Mann-Whitney U-test. *** P<0.001; ** P<0.01; ns: not significant (P≥0.05); HC: healthy control.

Correlations between cTfh and cTfr cell frequencies, B cell hyperactivity and disease activity

Next, we assessed whether cTfh or cTfr cell frequencies were associated with serological markers of B cell hyperactivity and systemic disease activity in pSS patients. Both cTfh and cTfr cell frequencies correlated significantly with serum levels of IgG and immunoglobulin free light chains (Figure 3 and data not shown). Frequencies of both cell subsets also correlated significantly with serum levels of CXCL13 (Figure 3). CXCL13 is an important chemokine for B cell homing to lymphoid follicles and is involved in ectopic lymphoid tissue formation in pSS [23,24]. On the other hand, the serum level of CXCL10, a biomarker that is associated with pSS[25], but not directly with B cell activity, was not significantly associated with cTfh or cTfr cell frequencies (data not shown). Together, these results indicate that increased frequencies of cTfh and cTfr cells are related to T cell-dependent B cell activity in pSS. Furthermore, these cell subsets correlated significantly with systemic disease activity, as measured by ESSDAI and clinESSDAI scores. (Figure 3).

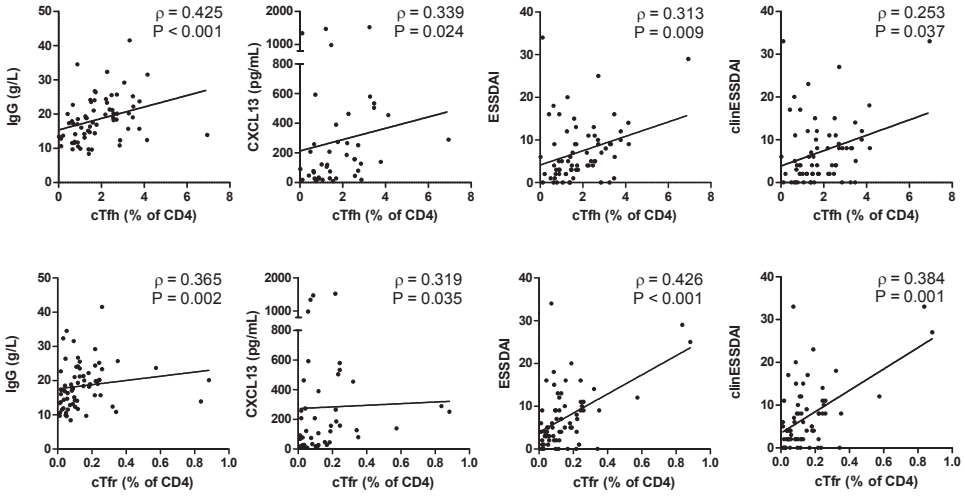

FIGURE 3 | Correlations between cTfh and cTfr cell frequencies, signs of B cell hyperactivity and disease activity in pSS. Frequencies of (A) circulating T follicular helper (cTfh) cells and (B) circulating T follicular regulatory (cTfr) cells were correlated with serum levels of IgG and CXCL13, and ESSDAI scores. Baseline data from pSS patients in the rituximab group (n=24) and data from the inception cohort (n=44) were combined. Correlations were evaluated with Spearman's correlation coefficient (ρ). ESSDAI: EULAR Sjögren's syndrome disease activity index.

CTLA-4 expression by cTfr cells and other regulatory T cells in pSS

In addition to markers for phenotypic classification of human $CD4^+$ T cells, median expression levels (median fluorescence intensity; MFI) of CTLA-4 were assessed. CTLA-4 is important for immune suppression and alterations in CTLA-4 expression may affect immune homeostasis in pSS. We observed that levels of CTLA-4 in peripherally induced Treg (pTreg) cells as well as cTfr cells were significantly lower in pSS patients, compared with HCs (Figure 4C). The highest difference in CTLA-4 expression between pSS patients and HCs was seen in activated cTfr cells ($CXCR5^+PD\text{-}1^+FoxP3^{high}$). These data suggest that the immune suppressive potential of pTreg cells, and in particular cTfr cells, is reduced in pSS patients. Since Tfr cells negatively regulate formation of Tfh cells [8], we correlated the MFI of CTLA-4 in cTfr cells to the frequency of cTfh cells. In patients with lower CTLA-4 expression levels, the frequency of cTfh cells tended to be higher (Figure 4D). The correlation between CTLA-4 expression levels by activated ($FoxP3^{hi}$) cTfr cells and frequencies of cTfh cells was even stronger (Spearman's ρ=0.60, P=0.002). Together, these findings indicate that the suppressive capacity of cTfr cells is possibly reduced in pSS patients with decreased CTLA-4 expression.

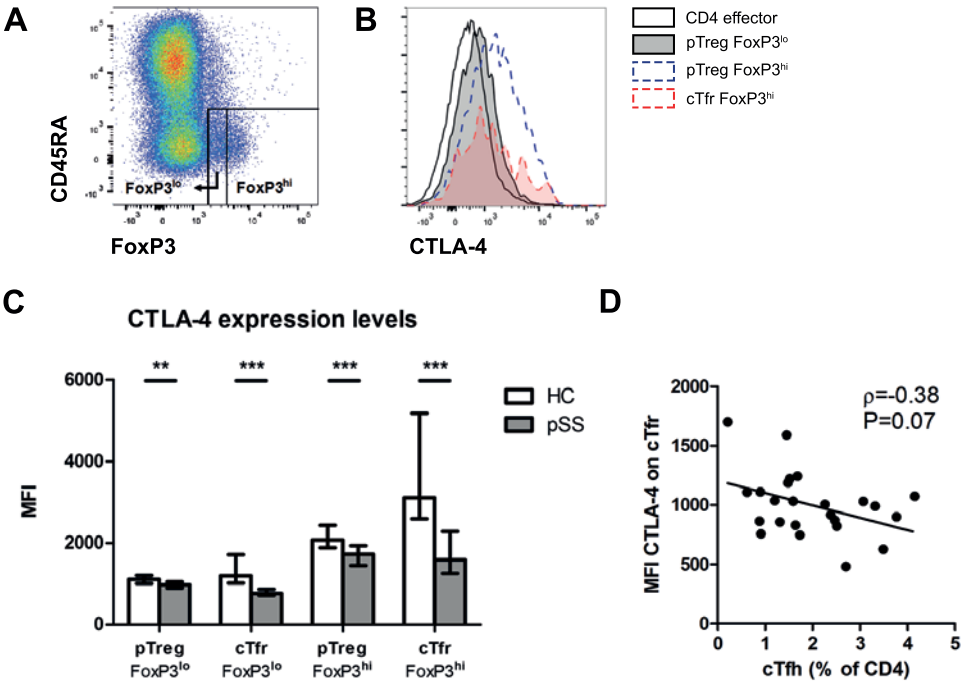

FIGURE 4 | Altered expression of CTLA-4 by regulatory T cells in pSS. (A) Representative dot plot of CD45RA and FoxP3 expression in $CD3^+CD4^+$ cells from a pSS patient. Regulatory cells were subdivided into $FoxP3^{lo}$ (resting) and $FoxP3^{hi}$ (activated). (B) Representative histogram of CTLA-4 expression levels in different $CD4^+$ cell subsets from a pSS patient. (C) Median fluorescence intensity (MFI) of CTLA-4 in resting and activated circulating pTreg cells ($CD45RA^-FoxP3^+CXCR5^-$) and circulating T follicular regulatory (cTfr) cells ($CD45RA^-FoxP3^+CXCR5^+PD\text{-}1^+$). Data from 24 pSS patients and 24 healthy controls (HC) are displayed. P-values were calculated using the nonparametric Mann-Whitney U-test. Bars indicate the median and interquartile range. (D) Correlation between CTLA-4 expression levels by cTfr cells and frequencies of circulating T follicular helper (cTfh) cells, evaluated with Spearman's correlation coefficient (ρ). *** P<0.001; ** P<0.01.

DISCUSSION

Tfh cells are elevated in the inflamed salivary glands and peripheral blood of pSS patients and are likely involved in the establishment of T cell-dependent B cell hyperactivity [14,15,26,27]. Tfr cells are important regulators of Tfh cell proliferation and B cell activation [28]. This study shows that not only cTfh, but also cTfr cell frequencies are increased in pSS patients compared with HCs, even to a larger extent than cTfh cells. A major proportion of cTfr cells in pSS patients expressed CXCR3, promoting CXCL10-driven migration to inflamed tissues. Frequencies of cTfr cells correlated positively with serological markers of B cell activity and with systemic disease activity (i.e., ESSDAI and clinESSDAI scores). The increased frequencies of cTfr cells coincided with reduced expression of CTLA-4 by these cells, indicating that their suppressive potential is affected.

Recently, Fonseca et al. also showed that cTfr cells were increased in pSS patients [27,29]. In contrast to our study, they defined cTfr cells as $CD4^+CXCR5^+Foxp3^+$ T cells, comprising both naive and memory T cells. They showed that the majority of cTfr cells were $CD45RA^+Foxp3^{lo}$ resting, naive-like Treg cells [29]. These cells were able to suppress effector T cell proliferation, but lacked full B cell-suppressive capacity [29]. The authors suggested that the increase in cTfr cells could be a result of ongoing T cell activation in secondary lymphoid organs, leading to an increased output of cTfr (and cTfh) cells. In our study, cTfr cells were defined as $CD4^+CD45RA^-FoxP3^+CXCR5^+PD\text{-}1^+$ T-cells, hereby selecting the smaller proportion of antigen-experienced, memory-like cells. In mice, these memory-like cTfr cells were shown to persist in the circulation for (at least) 30 days, with the capacity to home to GCs after T cell reactivation [16]. These memory-like cTfr cells are therefore likely also the cells that migrate to the inflamed glandular tissues. As a result of a greater rise in cTfr cells compared with cTfh cells, the cTfr/cTfh ratio is increased in pSS patients. In B cell follicles, a higher Tfr/Tfh ratio negatively regulates antibody responses [30]. However, immune responses are clearly not sufficiently suppressed in pSS. CTLA-4 is an important receptor for immune suppression by Treg cells. We found that expression levels of CTLA-4 are decreased in circulating pTreg cells, including cTfr cells, from pSS patients compared with HCs. As CTLA-4 reduces availability of CD80/86 on APCs [8], lower CTLA-4 levels may result in less efficient suppression of Tfh cell expansion and consequently increased B cell activity. Consistent with this notion, we observed a reverse correlation between cTfh cell frequencies and CTLA-4 expression levels by cTfr cells.

Lower expression of CTLA-4 by Treg cells may not only affect proliferation of effector cells, like Tfh cells, but also proliferation of the Treg population itself can be enhanced [31,32]. In line with this notion, in the experimental autoimmune encephalomyelitis (EAE) mouse model, CTLA-4 deletion in Treg cells from adult mice resulted in expansion of effector T cells as well as Treg cells. Interestingly, we observed previously that frequencies of pTreg cells are also increased in peripheral blood of pSS patients [15]. The work presented here suggest that lower CTLA-4 levels may contribute to this expanded pool of pTregs. The implications of higher pTreg and cTfr cell frequencies for immune suppression and disease activity in pSS still need to be elucidated. Furthermore, the origin of these cell subsets remains controversial. A recent study in mice showed that Tfr cells could be derived from either $FoxP3^+$ or $FoxP3^-$ precursor cells [33]. This study also showed that Tfr cells can be specific for self-antigen as well as foreign (immunized) antigen. However, another study in mice showed that Tfr cells were not specific for the immunized antigen, and instead expressed a TCR repertoire closely resembling that of thymus-derived natural Treg cells [34]. Because natural Treg cells have a TCR repertoire that is skewed towards self-antigen recognition [35], the latter study suggest that Tfr cells are important for the suppression of autoimmune responses. Whether the

expanded cTfr cell population in pSS patients recognizes mainly self-antigens or foreign antigens remains unknown and needs further investigation.

In summary, the expansion of cTfr cells in pSS is associated with increased B cell activity as well as systemic disease activity. Apparently, these cells do not exhibit (full) suppressive function and fail to control B cell responses once they are recruited to secondary lymphoid organs and/or ectopic lymphoid tissue in the inflamed glands. Although lower CTLA-4 expression is suggestive of lower suppressive potential, the functional capacity of memory-like cTfr cells needs to be investigated to confirm this assumption. Novel insights into the role of cTfr cells in pSS pathogenesis may lead to development of new therapies to enhance or mimic suppressive function of these cells and attenuate B cell activity. In addition, therapies that reduce the elevated levels of cTfh and pTreg cells in pSS patients, as we have shown for abatacept [15], may (partially) restore the disturbed immune homeostasis in this disease.

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