ATTENUATION OF FOLLICULAR HELPER T CELL–DEPENDENT B CELL HYPERACTIVITY BY ABATACEPT TREATMENT IN PRIMARY SJÖGREN’S SYNDROME

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ABSTRACT

Objective
To assess the effect of abatacept (CTLA-4Ig), which limits T cell activation, on homeostasis of CD4+ T cell subsets and T cell-dependent B cell hyperactivity in patients with primary Sjögren’s syndrome (SS).

Methods
Fifteen patients with primary SS treated with abatacept were included. Circulating CD4+ T cell and B cell subsets were analyzed by flow cytometry at baseline, during the treatment course, and after treatment was completed. CD4+ effector T cell subsets and Treg cells were identified based on expression of CD45RA, CXCR3, CCR6, CCR4, CXCR5, programmed death 1, inducible costimulator (ICOS), and FoxP3. Serum levels of anti-SSA/anti-SSB and several T cell-related cytokines were measured. Expression of ICOS and interleukin-21 (IL-21) protein was examined in parotid gland tissue at baseline and after treatment. Changes in laboratory parameters and associations with systemic disease activity (EULAR Sjögren’s Syndrome Disease Activity Index [ESSDAI]) over time were analyzed using generalized estimating equations.

Results
Abatacept selectively reduced percentages and numbers of circulating follicular helper T (Tfh) cells and Treg cells. Other CD4+ effector T cell subsets were unaffected. Furthermore, expression of the activation marker ICOS by circulating CD4+ T cells and expression of ICOS protein in parotid gland tissue declined. Reduced ICOS expression on circulating Tfh cells correlated significantly with lower ESSDAI scores during treatment. Serum levels of IL-21, CXCL13, anti-SSA, and anti-SSB decreased. Among circulating B cells, plasmablasts were decreased by treatment. After cessation of treatment, all parameters gradually returned to baseline.

Conclusion
Abatacept treatment in patients with primary SS reduces circulating Tfh cell numbers and expression of the activation marker ICOS on T cells. Lower numbers of activated circulating Tfh cells contribute to attenuated Tfh cell–dependent B cell hyperactivity and may underlie the efficacy of abatacept.
INTRODUCTION

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease characterized by dry eyes and mouth, associated with infiltration of lacrimal and salivary glands by T and B lymphocytes [1]. Patients with pSS may also have a diversity of extraglandular manifestations such as fatigue and musculoskeletal, lung, and skin involvement.

B cell hyperactivity is a prominent feature of pSS, reflected by hypergamma-globulinemia and autoantibody production, among others [2]. Consistent with this notion, we showed that B cells from pSS patients, including antigen-inexperienced naive B cells, express elevated levels of the B cell receptor signaling molecule Bruton’s tyrosine kinase (BTK) [3]. Elevated BTK levels have been linked to T cell-mediated autoimmune pathology in both rheumatoid arthritis (RA) and pSS, indicating that there is a pro-inflammatory feedback loop between B cells and CD4+ T cells [3]. Although in principle, all CD4+ effector T cell subsets can provide help to B-cells, T follicular helper (Tfh) cells are considered to be the most effective cells involved in T cell-dependent activation of B cells, leading to humoral immune responses [4]. Tfh cells facilitate generation of autoantibodies and formation and maintenance of germinal centers (GCs) [5]. The majority of Tfh cells are located in secondary lymphoid organs, where they characteristically express CXCR5, programmed death 1 (PD-1), inducible costimulator (ICOS), and the transcription factor Bcl-6 [5]. CD4+ T cells with a similar phenotype, called circulating Tfh (cTfh) cells, have been found in peripheral blood [6]. The transcriptional profile of cTfh cells resembles that of classical Tfh cells located in lymphoid organs, except that cTfh cells do not express Bcl-6 [7]. It remains to be elucidated whether cTfh cells are precursor cells of classical Tfh cells, derive from these cells, or both [8].

Recently, Rao et al [9] showed that another CD4+ T cell subset, defined as CXCR5-PD-1high and provisionally named peripheral helper T (Tph) cells, also promotes B cell responses and that this subset is pathologically expanded in RA patients. Like cTfh cells, Tph cells may functionally resemble classical Tfh cells. Upon activation, Tfh, cTfh, and Tph cells up-regulate costimulatory molecules essential for interaction with B cells, including PD-1 and ICOS [10,11]. In addition, all 3 subsets can secrete interleukin-21 (IL-21), which is critical for the support of B cell proliferation and differentiation [12].

Within the peripheral CD4+ T cell compartment of patients with B cell-mediated autoimmune diseases, increased frequencies of cTfh cells are commonly seen [5]. In patients with pSS, proportions of cTfh cells and serum levels of their signature cytokine IL-21 are increased [6,13-15]. Tfh cells may be present in the target tissue as well, since elevated protein levels of IL-21 have been found in minor salivary glands [13]. Although Tfh cells are a major source of IL-21, we cannot exclude the possibility that other cell types that produce IL-21, such as Th17 cells, natural killer T cells, and Tph cells, are at
least partly responsible for this increase in IL-21 levels [16]. Lowering of IL-21-producing T cells may reduce T cell-dependent B cell hyperactivity in patients with pSS and is therefore a feasible treatment option.

Abatacept is a fully human fusion molecule combining cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) with IgG Fc that binds to CD80/86 and consequently impairs CD28-mediated T cell costimulation [17]. As a result, T cell-dependent B cell activation may be reduced, both indirectly by inhibiting dendritic cell-mediated differentiation of naive T cells into CD4+ effector T cells and directly by blocking T cell-B cell interaction [18,19]. To date, 2 open-label studies have evaluated the efficacy of abatacept in pSS [20,21]. Adler et al [21] observed histologic, cellular, and serologic changes in response to treatment, including a decrease in local T regulatory (Treg) cells and serum gamma globulins. They also observed a small, but significant, increase in salivary gland function after treatment. The study by Meiners et al [20] showed that abatacept improves systemic disease activity and patient-reported symptoms, as measured by the EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI) and Patient Reported Index (ESSPRI). That study further showed that salivary gland function remained stable during treatment (24 weeks) and during follow-up after cessation of treatment (24 weeks). Although clinical effects seem promising, the effects of abatacept on human T cells in vivo and on T cell-dependent B cell hyperactivity in pSS remain elusive. Therefore, the aim of this study was to assess the effect of abatacept on the phenotypic distribution of circulating CD4+ T cell and B cell subsets as well as on T cell-dependent B cell hyperactivity. We show that the numbers of cTfh cells were predominantly affected by abatacept treatment. Importantly, ICOS expression on CD4+ T cells also declined, both in peripheral blood and parotid gland tissue. The decrease in ICOS expression on cTfh cells correlated significantly with the reduction in systemic disease activity, as measured by ESSDAI, over time.

PATIENTS AND METHODS

Study population

This was a prospective, single-center, open-label study of patients with early and active pSS (n=15) [20]. All patients fulfilled the American-European Consensus Group criteria for pSS [22]. Patients had not previously received any biologic disease-modifying antirheumatic drug, and immunosuppressants, including hydroxychloroquine and oral prednisone, were discontinued before inclusion. Baseline characteristics of the patients are displayed in Supplementary Table 1. In addition, 15 age- and sex-matched healthy individuals (mean ± SD age 45 ± 11 years; 12 women) were included as healthy controls. All patients and healthy controls provided written informed consent. The study was
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approved by the Medical Ethics Committee of the University Medical Center Groningen (METc2009.371).

**Study procedures**

Patients were treated with abatacept for 24 weeks (~10 mg/kg by intravenous infusion on days 1, 15, 29 and every 4 weeks thereafter) [20]. Follow-up was conducted at weeks 4, 12 and 24 (while receiving treatment) and weeks 36 and 48 (after completing treatment). Serum and EDTA and lithium-heparinized blood samples were obtained at baseline and during follow-up. Peripheral blood mononuclear cells (PBMCs) were immediately isolated from lithium-heparinized blood as previously described [23], cryopreserved using Nalgene Mr. Frosty boxes, and stored at -196°C. Parotid gland biopsy specimens were obtained at baseline and at week 24 of follow-up. Systemic disease activity was measured using ESSDAI and Clinical ESSDAI (ClinESSDAI; ESSDAI without the biological domain) [24,25].

**Assessment of circulating CD4+ T cell and B cell subsets**

Absolute numbers of circulating CD4+ T cells and B cells were measured in fresh EDTA blood samples using the MultiTest TruCount method (Becton Dickinson). Fresh blood samples were stained with antibodies directed against human CD3, CD4, CCR7 and CD45RO (Supplementary Table 2), lysed, washed, and analyzed by flow cytometry for the presence of naive (CCR7+CD45RO-), central memory (CCR7+CD45RO+), effector memory (CCR7-CD45RO+), and terminally differentiated (CCR7-CD45RO-) CD4+ T cells [26]. In a separate experiment, cryopreserved PBMCs were thawed, 2 million cells were fluorescence labeled for effector memory CD4+ T cell subset analysis, and 2 million cells were fluorescence labeled for B cell subset analysis (see Supplementary Table 2 for a list of the antibodies used). CD4+ effector memory T cell subsets were identified by chemokine receptor patterns (Supplementary Figure 1). One patient was excluded from CD4+ effector memory T cell analysis because of consistently aberrant CD45RA expression, which precluded analysis of memory subsets. Flow cytometric measurements were performed on a FACS LSRII flow cytometer (Becton Dickinson), and data were analyzed using Kaluza (Beckman Coulter) and FlowJo (Tree Star) software.

**Quantification of cytokines and anti-SSA/SSB IgG titers in serum**

Serum CXCL13 levels were quantified as part of a custom-made ProcartaPlex Multiplex Immunoassay (eBioscience), which also included interferon-γ (IFNγ), IL-4, IL-17, IL-22 and IL-10. Serum IL-21 levels were measured by an in-house enzyme-linked immunosorbent assay (ELISA) using purified anti-human IL-21 and biotin-conjugated anti-human IL-21
(eBioscience). Serum titers of anti-SSA and anti-SSB IgG were measured by an in-house ELISA using Ro52, Ro60 and La antigens (Euro-Diagnostica) and horseradish peroxidase (HRP)-conjugated goat anti-human IgG-Fc (Bethyl Laboratories).

**Immunohistochemistry**

For immunohistochemical staining of ICOS expression in salivary glands, formalin-fixed, paraffin-embedded sections (3 μm) of parotid gland tissue were incubated for 1 hour at room temperature with primary antibodies to ICOS (clone SP98; Thermo Scientific). Prior to incubation, heat-induced epitope retrieval (HIER) was performed using EDTA buffer (pH 8). Anti-ICOS antibodies were visualized using an HRP-linked detection system with diaminobenzidine (Dako), and sections were analyzed for staining using the Positive Pixel Count algorithm (version 9.1) in Aperio ImageScope (Aperio Technologies). Only foci were depicted for analysis, and the relative area stained within these foci was calculated by dividing the positive pixel count by the total pixel count. HIER was performed on consecutive sections using citrate buffer (pH 6), and sections were incubated overnight at 4°C with primary antibody to IL-21 (polyclonal; Novus biologicals) and the next day for 1 hour at room temperature with anti-CD4 (clone 1F6; Abcam). Two-color immunofluorescence visualization of staining was performed using Alexa Fluor 488-conjugated anti-mouse IgG and Alexa Fluor 594-conjugated anti-rabbit IgG tyramide SuperBoost kits (Thermo Scientific) according to the manufacturer’s protocol. Fluorescence images were acquired using a TissueFAX system and data were analyzed using TissueQuest software (TissueGnostics).

**Statistical analysis**

All results are presented as the median (interquartile range [IQR]). Mann-Whitney U tests were used to compare differences between patients and healthy controls. Generalized estimating equations (GEEs) with exchangeable correlation structure were used to analyze changes in laboratory parameters and associations with systemic disease activity over time within subjects [27]. Data from baseline up to week 24 were compared with baseline to assess changes while patients were receiving treatment. Data from week 24 to week 48 were compared with week 24 to assess changes when patients were no longer receiving treatment. Wilcoxon’s matched pairs tests were used to compare histologic parameters at baseline and week 24. P values less than 0.05 (2-tailed) were considered significant. Statistical analysis was performed using SPSS Statistics version 23 (IBM).
RESULTS

Abatacept affects the distribution of CD4+ T cell subsets

In patients with pSS, total numbers of circulating CD4+ T cells at baseline were ~35% lower than in healthy controls (median 0.59 x 10^9/liter [IQR 0.40-0.85] versus 0.89 x 10^9/liter [IQR 0.82-0.96] in healthy controls; P=0.022). Systemic disease activity was relatively high in our study population, and it has previously been shown that CD4+ T cell numbers are most strongly decreased in pSS patients with high ESSDAI scores [28]. Within the CD4+ T cell fraction, proportions of naive and terminally differentiated CD4+ T cells were not significantly altered in pSS patients at baseline compared with healthy controls (Figures 1A and C). Proportions of central memory CD4+ T cells were significantly decreased (Figure 1B), whereas proportions of effector memory cells were significantly increased (Figure 1D), in pSS patients compared with healthy controls. These results indicate that the pSS patients studied had a relatively expanded effector memory CD4+ T cell compartment, at the expense of central memory CD4+ T cells. Consistent with this finding, it has been shown that pSS patients have a higher proportion of HLA-DR+CD4+ T cells, indicating recent activation of T cells [28].

Abatacept treatment resulted in a slight increase in total numbers of CD4+ T cells (~5%) at week 4 only (data not shown). Proportions of effector memory CD4+ T cells, and to a lesser extent central memory CD4+ T cells, were significantly decreased in patients treated with abatacept (Figures 1B and D), while proportions of naive and terminally differentiated CD4+ T cells were significantly increased (Figures 1A and C). The distribution of these 4 differentiation subsets was, however, not entirely normalized to the distribution seen in healthy controls (Figure 1). Also, absolute numbers of effector and central memory CD4+ T cells were significantly reduced by abatacept (P=0.009 and P<0.001, respectively) (data not shown). These findings indicate that the two memory CD4+ T cell subsets in particular are affected by abatacept, illustrating the dependency of memory T cell formation or maintenance on CD28-mediated costimulation [29].

Abatacept specifically reduces cTfh and Treg cell numbers in peripheral blood

Next, the various effector subsets and peripheral Treg cells that are comprised within the peripheral CD4+ T cell fraction were studied in more detail. Baseline proportions of cTfh cells (CD45RA–CXCR5+PD-1+), Th17 cells (CD45RA–CXCR5–CXCR3–CCR4+CCR6+) and peripheral Treg cells (CD45RA–FoxP3+) were all significantly increased in pSS patients compared with healthy controls (Figure 2A). These results are consistent with our previous findings in a different study population [30]. The recently described Tph cells (CXCR5–PD-1^high), which functionally resemble Tfh cells and provide B cell help [9],
were also significantly increased in the peripheral blood of pSS patients compared with healthy controls (Figure 2A). Their overall frequencies and numbers, however, remained much lower than cTfh cells.

**FIGURE 1 | Abatacept treatment alters the distribution of CD4+ T cell differentiation subsets.** Proportions of naïve CD4+ T cells (CCR7+CD45RO−) (A), central memory CD4+ T cells (CCR7+CD45RO+) (B), terminally differentiated CD4+ T cells (CCR7 CD45RO−) (C), and effector memory CD4+ T cells (CCR7 CD45RO+) (D) in healthy controls (HCs) and in patients with primary Sjögren's syndrome during treatment and after completing treatment are shown. Circles represent individual subjects; horizontal lines show the median. Changes in frequencies over time within patients during treatment (weeks 4-24) compared with baseline (week 0), and after treatment (weeks 36 and 48) compared with week 24, were analyzed by generalized estimating equation.
To explore the activation state of effector T cell subsets, we also analyzed the expression levels of ICOS on these cells. All effector subsets from pSS patients expressed significantly higher levels of ICOS than did healthy controls (data not shown), with the highest relative increase in expression seen on cTfh cells (Figure 2B). Apparently, effector CD4+ T cells from pSS patients are in a hyperactive state, which enhances their capacity to interact with antigen-presenting cells. ICOS expression was also present on peripheral Treg cells, but no significant difference in expression level between pSS patients and healthy controls was observed.

To assess the extent to which abatacept modulates T cell activation in pSS, we examined different CD4+ effector memory subsets at various time points while patients were receiving treatment and after they had stopped treatment. The most pronounced effects of abatacept were seen on cTfh cells. Treatment resulted in a significant decrease in absolute numbers and proportions of this subset over time (Figures 2D and E). Absolute numbers and proportions of peripheral Treg cells also declined in patients treated with abatacept (P<0.001) (see Supplementary Figure 1), while absolute numbers and proportions of Th1, Th2, and Th17 cells were not significantly affected (Supplementary Figure 1). The decrease in cTfh cells during treatment was accompanied by normalization of ICOS expression levels on these cells to levels seen in healthy controls (Figures 2F and G). The decrease in ICOS levels was further reflected by a decrease in the proportion of CXCR5+PD-1+ICOS+CD4+ cells (Figure 2H). ICOS expression on other effector subsets and on peripheral Treg cells was also reduced (data not shown).

Although the proportions of Tph cells were also decreased in patients treated with abatacept (P=0.003 for week 0-24, by GEE), their absolute numbers only tended to decrease (P=0.074) (Figure 3B). Thus, Tph cells were less affected by abatacept than (PD-1high) cTfh cells (Figure 3C). This selective decrease in cTfh cells was reflected by an increased ratio of CXCR5+PD-1+Tph cells to CXCR5+PD-1high Tfh cells during treatment (Figure 3D). After patients stopped treatment, the numbers and proportions of cTfh cells, peripheral Treg cells, and Tph cells, as well as ICOS expression levels on all subsets, reverted to baseline levels, underlining their (partial) dependency on CD28 signaling.

Since cTfh cells were reduced by abatacept, and given the notion that Tfh cells support the formation of plasmablasts, this B cell subset might also be affected by treatment. Therefore, we analyzed the number of circulating CD19+ B cells and the proportion of plasmablasts (CD19+CD27++CD38++) over time. Whereas the total number of B cells remained unchanged, the proportion of circulating plasmablasts decreased significantly during treatment (P<0.001) (Figure 4F). A small decrease in IgD-IgM+ non-switched memory cells was also observed, while naive, IgD+ non-switched memory and switched memory B cells were not significantly affected (Supplementary Figure 2).
Reduced serum IL-21 and CXCL13 levels in pSS patients treated with abatacept

We subsequently analyzed serum levels of signature cytokines of the various effector CD4+ T cell subsets. IL-21, the signature cytokine of Tfh cells, was detectable in 12 patients at baseline (80%). In these patients, abatacept treatment significantly decreased IL-21 levels (Figure 4A). In addition to IL-21, Tfh cells can produce the homeostatic chemokine CXCL13. Serum levels of this chemokine were also significantly reduced by abatacept.
treatment (Figure 4B). After patients stopped treatment, serum levels of both IL-21 and CXCL13 returned to baseline values (Figures 4A and B). Signature cytokines of other CD4+ effector subsets were not significantly altered by treatment (IL-17 and IL-22) or remained undetectable in serum at all time points (IFNγ, IL-4, and IL-10), which may be a consequence of the sensitivity of the assay.

**FIGURE 3 |** Abatacept treatment affects numbers of peripheral helper T (Tph) cells less than numbers of PD-1high circulating follicular helper T (Tfh) cells in patients with primary Sjögren’s syndrome (pSS). A, Gating strategy for CXCR5PD-1high cells (red) and CXCR5PD-1high cells (blue) within the CD45RA CD4+ memory compartment. B and C, Absolute numbers of Tph cells (CXCR5PD-1high CD4+) (B) and PD-1high cTfh cells (CXCR5 PD-1high CD4+) (C) in patients with pSS during treatment and after completing treatment. D, Ratio of Tph cells to cTfh cells in patients with pSS during treatment and after completing treatment. In B-D, circles represent individual subjects; horizontal lines show the median. Changes over time within patients during treatment (weeks 4-24) compared with baseline (week 0), and after treatment (weeks 36 and 48) compared with week 24, were analyzed by generalized estimating equation. PD-1 = programmed death 1.
FIGURE 4 | Reduced circulating follicular helper T cell-related cytokines, autoantibodies and plasmablasts (PB) in patients with primary Sjögren’s syndrome (pSS) treated with abatacept. A, Serum interleukin-21 (IL-21) levels, measured by an enzyme-linked immunosorbent assay (ELISA), in 12 pSS patients during treatment and after completing treatment. B, Serum CXCL13 levels, measured by multiplex bead assay, in 15 pSS patients during treatment and after completing treatment. In A and B, circles represent individual patients; horizontal lines show the median. C-E, Serum concentrations of autoantibodies directed against Ro52 (C), Ro60 (D) and La (E), as measured by ELISA. The percent change was calculated using the baseline concentration as the reference. Values are the median (interquartile range). F, Left, Identification of plasmablasts as CD19⁺CD27⁺⁺CD38⁺⁺ cells. Middle and right, Number (middle) and proportion (right) of plasmablasts in pSS patients during treatment and after completing treatment. Circles represent individual patients; horizontal lines show the median. P values were determined by Mann-Whitney U test. Changes over time within patients during treatment (weeks 4-24) compared with baseline (week 0), and after treatment (weeks 36 and 48) compared with week 24, were analyzed by generalized estimating equation.
Abatacept affects SSA and SSB antibody titers

Since serum IL-21 levels, numbers of cTfh cells, and plasmablasts were all reduced by treatment, we next investigated whether concentrations of autoantibodies in serum were also affected by abatacept. Consistent with the observed decrease of ~20% in total IgG [20], levels of IgG antibodies to both isoforms of SSA (Ro52 and Ro60), and to SSB (La) were decreased to a similar extent during treatment (Figures 4C-E). Anti-Ro52 and anti-Ro60 levels, but not anti-La levels, increased significantly after cessation of treatment. Thus, abatacept limits maintenance of IgG production, including autoantibodies, which is likely a result of reduced Tfh cell-mediated activation of B cells.

FIGURE 5 | Diminished expression of inducible costimulator (ICOS) protein in parotid gland tissue from patients with primary Sjögren's syndrome (pSS) treated with abatacept. A, Left, Representative image of ICOS staining (brown), counterstained with hematoxylin, in parotid gland tissue from a patient with pSS at baseline. Boxed area shows a cell that is strongly positive for ICOS. Right, Positive pixel count analysis using Aperio ImageScope software, which discriminates negative (blue), weak positive (yellow), positive (orange), and strong positive (red) pixels. B, Quantification of total positive staining and strong positive staining for ICOS within periductal foci at baseline and week 24. Differences in staining between baseline and week 24 were analyzed by Wilcoxon's matched pairs test.
Decreased ICOS expression in the parotid glands of patients treated with abatacept

To explore whether the effects on cTfh cells were also reflected in the inflamed glandular tissue, we analyzed protein expression of ICOS and IL-21 in the parotid gland tissue of patients with pSS before and after treatment. We recently showed that focus score and absolute numbers of CD3+ T cells/mm² parenchyma were not altered by treatment [31]. In the present study, immunohistochemical staining revealed that ICOS expression within foci was significantly decreased by treatment (Figure 5). Different levels of staining (weak, medium and strong) could be distinguished at baseline; after treatment, strong staining was nearly absent (Figure 5B). In contrast to ICOS expression, numbers of CD4+IL-21+ cells in parotid glands were not significantly affected by treatment (data not shown).

Correlation of changes in laboratory parameters with systemic disease activity

ESSDAI and ClinESSDAI were used to assess the clinical relevance of the Tfh cell-related changes observed during abatacept treatment. The decline in ICOS expression levels on cTfh cells, but not numbers of cTfh cells, was significantly associated with the decrease in ESSDAI and ClinESSDAI scores (Figure 6). Changes in serum levels of CXCL13, anti-Ro60 and rheumatoid factor (RF) induced by abatacept over time were also significantly associated with a decrease in ESSDAI and ClinESSDAI, whereas changes in the number of peripheral Treg cells and levels of total IgG, IL-21, anti-Ro52, and anti-La were not (Figure 6).

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<th>RF</th>
<th>anti-Ro60</th>
<th>CXCL13</th>
<th>Tfh (nrs)</th>
<th>pTreg (nrs)</th>
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FIGURE 6 | Associations between changes in systemic disease activity and systemic laboratory parameters over time in patients with primary Sjögren’s syndrome treated with abatacept.

Associations between systemic disease activity, as measured by the European League Against Rheumatism (EULAR) Sjögren’s syndrome Disease Activity Index (ESSDAI) and Clinical ESSDAI (ClinESSDAI; the ESSDAI without the biological domain), and systemic laboratory parameters that change over time during abatacept treatment (weeks 0-24) were analyzed by generalized estimating equation. The dependent variables (ESSDAI and ClinESSDAI) were square root transformed before they were entered into the equation. P values less than 0.005 were considered significant after Bonferroni correction. Rheumatoid factor (RF) levels were measured in kIU/liter. ICOS = inducible costimulator; MFI = median fluorescence intensity; Tfh = follicular helper T cell; nrs = number of cells; pTreg = peripheral Treg cells; IL-21 = interleukin-21; NS = not significant.
DISCUSSION

Previously, we showed in an open-label study that abatacept is effective for the treatment of pSS [20]. In the present study, we demonstrate that abatacept predominantly targets cTfh cells, which may help to explain the underlying mechanism of clinical efficacy. Abatacept resulted not only in a decrease in the numbers and activation state of cTfh cells, but also in reduced serum levels of Tfh cell-related cytokines (i.e., IL-21 and CXCL13). A decrease in cTfh cells likely contributed to the observed decrease in circulating plasmablasts and anti-SSA/SSB titers. Furthermore, abatacept reduced the numbers of peripheral Treg cells and tended to reduce the numbers of the novel Tph cell subset, defined as CXCR5-PD-1<sup>high</sup>, but did not affect Th1, Th2, or Th17 cells. Taken together, these findings indicate that the effects of abatacept are selective and reduce Tfh cell activity, thereby attenuating Tfh cell-dependent B cell hyperactivity in pSS patients.

Although the number of patients studied was small (n=15), baseline proportions of cTfh cells were significantly elevated in the patients compared with healthy controls. These results are consistent with previous findings in pSS [6,14,32]. A new observation is that cTfh cells from pSS patients have a 3-fold higher expression level of ICOS compared to healthy controls. Since ICOS is highly up-regulated upon activation [11,33], together with CXCR5 and PD-1, Tfh cells in patients with pSS are likely in a hyperactive state. Increased ICOS expression by CXCR5<sup>+</sup>CD4<sup>+</sup> T cells provides a stronger costimulatory signal to the B cell via ligation of ICOSL [34,35]. In turn, this interaction promotes expression of IL-21 by Tfh cells [36]. It is plausible that ICOS/ICOSL-mediated cross-talk between Tfh cells and B cells is a critical step in pSS-associated B cell hyperactivity. The importance of a positive-feedback loop between B cells and T cells in autoimmune pathology is illustrated by findings that BTK overexpression in B cells leads to systemic autoimmunity in mice, together with higher numbers of splenic Tfh cells and higher expression levels of ICOS on T cells, compared with wild-type mice [37].

Importantly, we demonstrate a selective decrease in the numbers and proportions of cTfh cells in pSS patients treated with abatacept, to levels normally seen in healthy controls. Furthermore, we showed that activation of cTfh cells, as revealed by ICOS expression, was strongly diminished by this treatment. In addition to its direct role in B cell activation, ICOS expression is essential for the maintenance and accumulation of Tfh cells [38,39]. Normalization of ICOS levels in pSS patients by abatacept, as a result of less CD28-mediated activation, may therefore be the key event leading to fewer Tfh cells. In RA patients, a decrease in cTfh cells has also been observed after abatacept treatment [40]. In the latter study, only one time point after treatment was evaluated, and expression of ICOS and other possible activation markers were not measured.

From animal studies it has become clear that abatacept limits activation of CD4<sup>+</sup> T cells after antigen exposure [19,41]. More specifically, it also prevents antigen-induced
expression of ICOS and PD-1 on CD4+ T cells, and autoantibody formation in mice [41]. The latter observations are consistent with the decrease in total IgG and RF observed in our cohort of abatacept-treated pSS patients [20] as well as with the decline in ICOS expression on CD4+ T cells, plasmablasts and anti-SSA and anti-SSB titers observed in the present study. Abatacept-treated patients with RA in remission also have reduced serum titers of autoantibodies [42]. It has been proposed that abatacept blocks survival signals to CD28-expressing autoreactive plasma cells, leading to apoptosis and consequently also to a decline in autoantibody titers [43,44]. However, we were unable to detect CD28+ plasma cells in parotid gland tissue from pSS patients (Kroese FGM: unpublished observations). The other explanation is that at least some of the autoantibody-producing plasma cells are relatively short-lived cells, which are not replaced by newly generated plasma cells in the absence of T cell help. In support of this notion, anti-Ro60 clonotypes in the serum of pSS patients are continuously replaced, which suggests that at least part of these IgG autoantibodies are derived from short-lived plasma cells [45].

In addition to the effects on cTfh cells, abatacept reduces numbers and proportions of peripheral Treg cells. This can be explained by the requirement of CD28 signaling for peripheral Treg survival and proliferation [46]. Consistent with our findings, Adler et al [21] observed a decrease in CD3+FoxP3+ cell numbers in labial salivary gland sections from pSS patients after abatacept treatment. It is likely that the peripheral and local decrease in peripheral Treg cells is an effect of less CD4+ T cell activation. Since the suppressive capacity of the remaining Treg cells is not known, the functional implication of this effect is not clear.

Low numbers of Tfh cells (CD3+Bcl6+) have also been detected in minor salivary glands of pSS patients [47]. Here they occur in close association with Bcl6+ GC B cells. In our study population, Bcl6+ cells were rarely detected in parotid gland tissue sections in the absence of GCs (data not shown). Since ICOS is highly expressed on Tfh cells and IL-21 is the signature cytokine of Tfh cells, we alternatively analyzed the presence of ICOS+ cells and CD4+IL-21+ cells by immunohistochemistry/immunofluorescence in parotid gland tissue before and after treatment with abatacept. ICOS staining was significantly lowered by treatment, and strongly ICOS-positive cells, which may represent Tfh cells, were nearly absent after treatment. As mentioned above, these reduced levels may result in less efficient costimulation of antigen-presenting cells in salivary gland tissue. Tfh cells in follicles require continuous signals from B cells via ICOS/ICOSL ligation. When ICOSL is blocked, Tfh cells lose CXCR5 expression and migrate back to the T cell zone, which results in a collapse of GCs [38]. Interestingly, in our study population a decrease in GCs in parotid gland tissue was seen after abatacept treatment [31].

Although ICOS expression in parotid gland tissue is reduced by treatment, the number of CD4+IL-21+ cells does not change significantly. Apparently, CD4+ T cells that reside in the gland are still activated by antigen-presenting cells and stimulated
to produce cytokines via costimulatory pathways other than CD28-CD80/86 and ICOS/ICOSL, or IL-21-production is maintained by autocrine regulation and/or continuous presence of IL-6. Possibly, IL-21 is still produced after abatacept treatment by other cells than classic ICOS\textsuperscript{high} Tfh cells, since IL-21 production is not restricted to Tfh cells [16]. For example, IL-21-producing CXCR5 PD-1\textsuperscript{high} CD4\textsuperscript{+} T cells were abundant in joint tissue of RA patients, and their frequency was higher than PD-1\textsuperscript{high}CXCR5\textsuperscript{+} CD4\textsuperscript{+} Tfh cells [9]. In addition, a novel population of \textit{Il21}\textsuperscript{+} Th1 cells was recently identified in salivary gland tissue from NOD mice [48], a murine model of SS, and this population may also be responsible for glandular IL-21 production in pSS patients.

In the present study, we showed that the decline in ICOS expression levels on cTfh cells was significantly associated with the decrease in systemic disease activity. Changes in the levels of CXCL13 and autoantibodies (RF and anti-Ro60) were also associated with a decrease in systemic disease activity. It is known that positivity for RF and anti-SSA, of which anti-Ro60 usually makes up the largest part, are both associated with the presence of extraglandular symptoms [49]. Also, serum levels of CXCL13 have been associated with systemic disease activity in pSS [50]. Attenuation of the hyperactivated state of Tfh cells by abatacept, with a concomitant decrease in serum RF, anti-SSA, and CXCL13 levels, probably underlies the amelioration of systemic disease activity. How these factors influence systemic disease activity is not yet understood and remains to be elucidated.

In contrast, the presence of glandular IL-21-producing CD4\textsuperscript{+} T cells was not affected by treatment in this study. Perhaps patients should be treated for a longer period of time to significantly reduce the influx of newly formed effector T cells into the glands. Alternatively, simultaneous blockade of other costimulatory pathways or direct targeting of IL-6 or IL-21 could be of additional value to target Tfh cell activity in the glands. In conclusion, abatacept affects cTfh cell activation and ICOS expression and concomitantly results in amelioration of disease activity. Our findings support the notion that Tfh cell-dependent B-cell hyperactivity plays a central role in pSS pathogenesis. We postulate that the cross-talk between Tfh and B cells is a key target for successful therapeutic interventions in pSS.

ACKNOWLEDGEMENTS

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REFERENCES


SUPPLEMENTARY MATERIALS

SUPPLEMENTARY TABLE 1 | Baseline characteristics of pSS patients.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>pSS patients (n=15)</th>
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<tr>
<td>Age, median (IQR), years</td>
<td>43 (32-51)</td>
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<tr>
<td>Female gender, n (%)</td>
<td>12 (80)</td>
</tr>
<tr>
<td>Disease duration (months), median (IQR)</td>
<td>11 (7-36)</td>
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<tr>
<td>ESSPRI, median (IQR)</td>
<td>7.5 (6-8)</td>
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<tr>
<td>ESSDAI, median (IQR)</td>
<td>11 (8-14)</td>
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<tr>
<td>clinESSDAI, median (IQR)</td>
<td>11 (9-17)</td>
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<td>IgG (g/L), median (IQR)</td>
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<td>Anti-Ro/SSA positive, n (%)</td>
<td>15 (100)</td>
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<tr>
<td>Anti-La/SSB positive, n (%)</td>
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<td>RF (kIU/L), median (IQR)</td>
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<td>SWS (mL/minute), median (IQR)</td>
<td>0.31 (0.02-1.47)</td>
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<tr>
<td>Focus score, median (IQR)</td>
<td>3.1 (1.5-5.0)</td>
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</table>

ESSPRI, European League Against Rheumatism (EULAR) Sjögren’s syndrome Patient Reported Index; ESSDAI, European League Against Rheumatism (EULAR) Sjögren’s syndrome Disease Activity Index; RF, rheumatoid factor; SWS, stimulated whole saliva. Focus score in parotid gland tissue.

SUPPLEMENTARY TABLE 2 | Antibodies used for flow cytometry.

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SUPPLEMENTARY FIGURE 1 | Effect of abatacept treatment on circulating pTreg-cells, Th17-cells, Th1-cells and Th2-cells. (A) Gating strategy for peripheral Treg (pTreg)-cells, Th17-cells, Th1-cells and Th2-cells in a representative healthy control and patient sample. First, pTreg-cells (CD3+CD4+CD45RA-FoxP3+) and FoxP3+ memory CD4+ T-cells were gated (CD3+CD4+CD45RA-FoxP3-). Th17-, Th1- and Th2-cells were distinguished based on the expression pattern of surface chemokine receptors. Fluorescence minus one (FMO) controls were used to set the gates. (B) Dot plots of the proportion of pTreg-cells, Th17-cells, Th1-cells and Th2-cells within CD4+ T-cells over time are displayed. Horizontal lines indicate the median. Changes over time within patients were analyzed by generalized estimating equations on treatment (week 0-24) and off treatment (week 24-48), compared with baseline and week 24, respectively. P-value < 0.05 was considered statistically significant.
SUPPLEMENTARY FIGURE 2 | Frequencies of circulating B-cell subsets in pSS patients at baseline, on treatment and off treatment. (A) Absolute numbers of circulating B-cells, (B) frequencies of naïve B-cells (CD27 IgD+), (C) frequencies of IgD+IgM non-switched memory B-cells (CD27 ‘IgD’), (D) frequencies of IgD+IgM+ non-switched memory B-cells (CD27 ‘IgD’IgM’) and (E) frequencies of switched memory B-cells (CD27 ‘IgD’IgM) are displayed. Horizontal lines indicate the median. If the data followed a linear course, changes over time within patients were analyzed by generalized estimating equations on treatment (week 0-24) and off treatment (week 24-48), compared with baseline and week 24, respectively. P-value < 0.05 was considered statistically significant.