T cell-dependent B cell hyperactivity in primary Sjögren's syndrome
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ENHANCED BRUTON’S TYROSINE KINASE ACTIVITY IN PERIPHERAL BLOOD B LYMPHOCYTES FROM PATIENTS WITH AUTOIMMUNE DISEASE

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ABSTRACT

Objective
Bruton's tyrosine kinase (BTK) transmits crucial survival signals from the B cell receptor (BCR) in B cells. Pharmacologic BTK inhibition effectively diminishes disease symptoms in mouse models of autoimmunity; conversely, transgenic BTK overexpression induces systemic autoimmunity in mice. We undertook this study to investigate BTK expression and activity in human B cells in the context of autoimmune disease.

Methods
Using intracellular flow cytometry, we quantified BTK expression and phosphorylation in subsets of peripheral blood B cells from 30 patients with rheumatoid arthritis (RA), 26 patients with primary Sjögren’s syndrome (pSS), and matched healthy controls.

Results
In circulating B cells, BTK protein expression levels correlated with BTK phosphorylation. BTK expression was up-regulated upon BCR stimulation in vitro and was significantly higher in CD27+ memory B cells than in CD27-IgD+ naive B cells. Importantly, BTK protein and phospho-BTK were significantly increased in B cells from anti-citrullinated protein antibodies (ACPA)-positive RA patients but not in B cells from ACPA-negative RA patients. BTK was increased both in naive B cells and in memory B cells and correlated with frequencies of circulating CCR6+ Th17 cells. Likewise, BTK protein was increased in B cells from a major fraction of patients with pSS and correlated with serum rheumatoid factor levels and parotid gland T cell infiltration. Interestingly, targeting T cell activation in patients with pSS using the CTLA-4Ig fusion protein abatacept restored BTK protein expression in B cells to normal levels.

Conclusion
These data indicate that autoimmune disease in humans is characterized by enhanced BTK activity, which is linked not only to autoantibody formation but also to T cell activity.
INTRODUCTION

B lymphocytes play a crucial role in various systemic autoimmune diseases. This is evident from the characteristic autoantibody repertoire, the genetic associations identified, and the promising efficacy of B cell-targeted therapies in rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and Sjögren’s syndrome (SS). Autoantibodies directed against nuclear self antigens in SLE and SS or anti-citrullinated protein antibodies (ACPAs) in RA often appear in patient serum before the onset of clinical symptoms [1–3].

In primary SS (pSS) [4], the presence of circulating nuclear autoantibodies and germinal centers (GCs) in salivary glands correlates with disease severity [5–7]. Moreover, genetic studies have implicated several genes involved in activation and differentiation of B cells in the pathogenesis of pSS and SLE, including BLK, BANK1, LYN and BAFF [1,8,9]. Next to being precursors of plasmablasts and plasma cells that secrete autoantibodies, B cells can engage T cells by supporting follicular Th cell differentiation, antigen presentation, production of inflammatory cytokines, and induction of tertiary lymphoid structures [10,11]. Although effects of B cell-depleting regimens in pSS are inconclusive [12–14], the efficacy of neutralizing BAFF in SLE and the efficacy of rituximab (an anti-CD20 antibody that eliminates B cells) in RA indicate that intelligent modulation of B cell function or survival may be key to successful treatment of systemic autoimmunity [1,15].

Promising drug candidates for autoimmune diseases include newly developed inhibitors of Bruton’s tyrosine kinase (BTK), which is a pivotal signaling molecule that directly links B cell receptor (BCR) signals to B cell proliferation and survival through activation of the transcription factor NF-κB [16]. The importance of BTK signaling to B cells is evident from the severe B cell deficiency in patients with X-linked agammaglobulinemia, who have mutations in the BTK gene [16,17]. The BTK small-molecule inhibitors ibrutinib and acalabrutinib have shown robust antitumor activity and limited adverse effects in clinical studies in patients with various B cell malignancies [18,19]. In murine models of SLE and RA, promising results have been obtained with BTK inhibition, which could prevent or ameliorate lupus nephritis or joint inflammation by correcting BCR-mediated B cell activation and autoantibody production [20–26] and also by dampening myeloid cell activation [18,24–26].

In the mouse, BTK protein expression levels in naive B cells are rapidly up-regulated upon BCR engagement or when B cells are activated by Toll-like receptor (TLR) or anti-CD40 stimulation [27,28]. Several mechanisms are involved in this positive feedback regulation, including microRNA-185 and NF-κB signaling [29,30]. Sufficient BTK expression is crucial for normal B cell development and function in mice [31,32]. On the other hand, appropriate regulation of BTK protein expression in B cells is crucial for maintaining immune tolerance, because CD19-hBTK-transgenic mice (with modest B cell-restricted human BTK overexpression under control of the CD19 promotor)
spontaneously develop SLE/pSS-like disease pathology [28]. In these mice, B cells are resistant to apoptosis, which aids their differentiation into autoantibody-producing plasma cells [28]. CD19-hBTK-transgenic mice manifest spontaneous GC formation, anti-nuclear autoantibodies, and lymphocyte infiltration in various organs, including salivary glands [28]. We recently found that BTK overexpression in B cells disrupts T cell homeostasis and promotes follicular Th cell differentiation, both in aging mice and in a collagen-induced arthritis model [33].

The finding that a modest increase in BTK expression in B cells is sufficient to induce systemic autoimmune disease in mice prompted us to examine BTK expression levels and regulation in B cell subsets in peripheral blood of healthy controls and patients with autoimmune disease. We show that human B cells also up-regulate BTK protein levels upon activation, and we provide data on aberrant expression levels and phosphorylation status of BTK in B cells from ACPA-positive RA patients. Likewise, we found that BTK protein was increased in B cells from a majority of patients with pSS.

**PATIENTS AND METHODS**

**Patients and healthy individuals**

Data on characteristics of the patients and healthy controls are summarized in Supplementary Table 1.

**RA patients**

Cohorts of ACPA-positive and ACPA-negative treatment-naïve patients with early RA who were matched for the Disease Activity Score (DAS) in 44 joints [34], the presence of rheumatoid factor (RF), and the duration of symptoms have been described previously [35]. All patients met the American College of Rheumatology/European League Against Rheumatism (EULAR) 2010 classification criteria for RA [36]. Fifteen ACPA-positive and 15 ACPA-negative patients were included and matched with 15 healthy controls.

**Patients with pSS**

We included 26 patients with pSS who were naïve to treatment with biologic disease-modifying antirheumatic drugs (DMARDs) and who fulfilled the American-European Consensus Group criteria for SS [37]; we matched these patients with 26 healthy controls. Fifteen of the patients with pSS had participated in the previously reported Active Sjögren Abatacept Pilot (ASAP) study (METc2009.371) [38]. They had been treated with intravenous abatacept on days 1, 15, and 29 and every 4 weeks thereafter until week 24, a regimen that improved disease activity as previously reported [38]. Patients were not treated with DMARDs or prednisone for at least 1 month prior to or during this study.
Serum and peripheral blood mononuclear cells (PBMCs) were collected from all healthy controls and patients at baseline (untreated) and from the 15 abatacept-treated patients with pSS at 4, 12, 24, 36, and 48 weeks after the first dose. Experimental procedures were approved by the Erasmus Medical Center and University Medical Center Groningen medical ethics committees. All patients provided written informed consent.

**Isolation and culture of human peripheral blood B cells**

PBMCs were isolated by standard Ficoll-Paque (GE Healthcare) density gradients. Subsequent purification of naive B cells was performed using a human Naive B Cell Isolation Kit II (Miltenyi Biotec), and B cell purity (>95%) was verified using flow cytometry. B cells were cultured in the presence of 10 µg/mL F(ab')\(_2\) goat anti-mouse IgM (Jackson ImmunoResearch), 2 µg/mL recombinant CD40L (R&D Systems), or 2 ng/mL lipopolysaccharide (LPS) for 3 days.

**Flow cytometry procedures**

Fluorescence labeling of cells and measurement of intracellular BTK levels were performed as described previously [28] (see Supplementary Table 2 for a list of the antibodies used). BTK gate settings were based on isotype controls, fluorescence minus one controls, and analysis of T cells, which lack BTK expression. For staining of phosphorylated BTK, PBMCs were left unstimulated or were stimulated for 30 seconds with F(ab')\(_2\) anti-human IgM (20µg/mL; SouthernBiotech) and subsequently fixed with Cytofix and permeabilized with Phosflow Perm Buffer III (BD Biosciences). Flow cytometric measurements were performed on an LSRII flow cytometer (BD Biosciences), and data were analyzed using FlowJo software (Tree Star).

**Immunohistochemistry**

Parotid gland biopsy specimens were obtained from 15 patients with pSS at baseline, and paraffin-embedded sections were stained with rabbit anti-human CD3 antibody (Ventana Medical Systems) and counterstained with hematoxylin using standard procedures. Numbers of CD3\(^+\) cells/mm\(^2\) were analyzed using HistoQuest software.

**Laboratory assessments in serum**

Baseline levels of serum immunoglobulin classes and RF were measured by nephelometry.
Statistics
Significance of continuous data was calculated using the nonparametric Mann-Whitney U test or one-way analysis of variance (ANOVA) or repeated-measures ANOVA with Tukey’s multiple comparison test. P values less than 0.05 were considered significant. Significance of correlations was determined with a nonparametric Spearman test. To determine significance of the effect of abatacept on BTK levels in B cells within patients over time, a generalized estimating equation was performed using SPSS statistical software (IBM).

RESULTS
BTK protein expression is up-regulated in in vitro-activated human B cells and correlates with BTK phosphorylation
Upon BCR stimulation of human B cells, a signaling cascade is initiated whereby BTK is phosphorylated in its kinase domain at position Y551 [18]. To verify this, we stimulated fractions of PBMCs from healthy controls with anti-IgM in vitro and found induction of phosphorylated BTK in B cells by phosphoflow analysis (Figure 1B).

Parallel to the findings in murine B cells [27,28], we observed that anti-IgM stimulation in vitro also induced an upregulation of BTK protein expression in human B cells, as detected by intracellular flow cytometry; average mean fluorescence intensity values increased ~1.4-fold (Figures 1A and C). Other stimuli, including recombinant CD40L, LPS, and imiquimod (a TLR-7 agonist), induced a limited but consistently detectable increase in BTK protein levels (Figure 1A and data not shown). To further study the relationship between BTK protein expression and phosphorylation of BTK at Y551 in human B cells, we measured BTK protein and phospho-BTK in gated CD19+ B cells from unstimulated fractions of PBMCs from 20 subjects. Signals for BTK protein and phospho-BTK varied considerably between individuals, but these were strongly correlated in individual subjects (Figure 1D and E). From these findings, we conclude that BTK protein expression levels are a sensitive and functional indicator of BTK activity in human B cells.

Differential expression of BTK protein in individual peripheral blood B cell subsets
In contrast to phosphoflow analysis, which is difficult to perform in conjunction with cell surface markers, the staining procedure for total BTK protein allows for quantification of BTK expression levels in individual B cell subsets. This enabled us to compare BTK expression between subsets of unstimulated peripheral blood B cells from healthy controls ex vivo (gating strategy is shown in Figure 2A). We observed that BTK expression
levels were significantly higher in antigen-experienced CD27⁺IgD⁺ and CD27⁺IgD⁻IgM⁺ nonswitched memory B cells compared with CD27⁺IgD⁺ naive B cells and CD27⁺IgD⁻IgM⁻ switched memory B cells (Figures 2B and C). These data indicate that BTK protein levels are tightly regulated during B cell differentiation and suggest that up-regulation of BTK expression in specific B cell subsets may have a physiologic function.

**FIGURE 1** | Bruton’s tyrosine kinase (BTK) protein expression and phosphorylation in human B cells are correlated and up-regulated upon B cell receptor stimulation in vitro. A, Representative graph of intracellular BTK expression in magnetic-activated cell-sorted B cells from a healthy control after 3 days of stimulation with anti-IgM (10 mg/ml), recombinant CD40L (rCD40L; 2 mg/ml), or lipopolysaccharide (LPS; 2 ng/ml). B, Representative graph of BTK phosphorylation in healthy control B cells that were either left unstimulated or were stimulated for 30 seconds with anti-IgM (20 mg/ml). C, BTK expression in magnetic-activated cell-sorted B cells from healthy controls that were either left unstimulated or were stimulated for 3 days with anti-IgM (10 mg/ml). Symbols represent specimens from individual subjects. D, BTK protein and phospho-BTK expression in B cells from healthy controls expressing high or low levels of BTK at baseline. E, Correlation between BTK protein and phospho-BTK expression in B cells from 20 subjects. Data are representative of 2 individual experiments. MFI = mean fluorescence intensity.
To investigate BTK activity in the context of autoimmune disease, we studied peripheral blood B cells from 15 ACPA-positive and 15 ACPA-negative treatment-naive RA patients who were matched for the DAS, and we included 15 age/sex-matched healthy controls (patient characteristics at baseline are displayed in Supplementary Table 1). The proportions of total B cells and the distribution over naive and various memory B cell subsets were not significantly different between RA patients and healthy controls (Figure 3A). Interestingly, when we quantified BTK expression in total B cells, we found that BTK protein levels were significantly increased (~1.4-fold) in ACPA-positive RA patients compared with ACPA-negative RA patients and healthy controls (Figure 3B) (representative flow cytometry dot plots are displayed in Supplementary Figure 1).
FIGURE 3  |  Increased expression of Bruton’s tyrosine kinase (BTK) in B cells from anti-citrullinated protein antibody (ACPA)-positive patients with rheumatoid arthritis (RA).  

A, Proportions of total B cells among live cells and proportions of naive B cells, IgD+ and IgD- nonswitched memory B cells, and switched memory B cells among total B cells in RA patients and healthy controls (HC). B, BTK protein expression in total B cells. Representative flow plots show BTK protein expression in an ACPA-positive patient, an ACPA-negative patient, and a healthy control. C, Phospho-BTK expression in total B cells. Representative flow plots show phospho-BTK expression in an ACPA-positive patient, an ACPA-negative patient, and a healthy control. D, Correlation between BTK protein and phospho-BTK expression in ACPA-positive patients (squares), ACPA-negative patients (triangles), and healthy controls (circles). E, BTK protein expression in the indicated B cell subsets in RA patients and healthy controls. Symbols represent specimens from individual subjects; bars show the median. MFI = mean fluorescence intensity. BTK expression levels in B cells correlate with frequencies of circulating CCR6+ Th17 cells.

Similar results were found for phospho-BTK levels in unstimulated B cells, consistent with the significant correlation observed between total BTK and phospho-BTK signals (Figures 3C and D). Compared with ACPA-negative RA patients and healthy controls, ACPA-positive RA patients showed increased BTK protein expression both in CD27- IgD+ naive B cells and in all the individual CD27+ memory B cell subsets (Figure 3E). In ACPA-positive RA patients, BTK was differentially expressed between different B cell subsets, as observed in healthy controls (Supplementary Figure 2). Taken together, our intracellular flow cytometry analyses demonstrate that in ACPA-positive RA patients,
but not in ACPA-negative RA patients, relative BTK expression values were increased in all B cell subsets, including naive and memory B cells, compared with healthy controls.

Since we found that BTK-overexpressing B cells in transgenic mice have the capacity to disrupt T cell homeostasis [33], we wondered whether BTK expression levels in RA patients correlated with parameters of T cell activity. We found that BTK protein levels correlated significantly with frequencies in peripheral blood of Th17-lineage cells, which have recently been implicated in RA etiology [39] (Figures 4A and B) (gating strategy is displayed in Supplementary Figure 3). Although BTK protein levels and frequencies of follicular helper T (Tfh) cells did not correlate significantly (Figure 4C), we did observe a positive correlation of BTK protein levels with inducible costimulator (ICOS) expression on Tfh cells (Figure 4D). The latter finding is interesting, because we previously found that in mice overexpressing BTK in B cells, the expression of ICOS on T cells, including Tfh cells, was increased [33]. No correlation was found with other T cell subsets, including Th1 and Treg cells (data not shown).

In summary, the finding that BTK protein levels correlate with the frequencies of Th17-lineage cells and with Tfh cell ICOS expression suggests that BTK activity is linked to T cell activation in RA.

Peripheral blood BTK protein expression is increased in a major fraction of patients with pSS

To explore whether increased BTK activity is unique to RA or can also be found in other autoimmune disorders, we investigated BTK expression in 26 treatment-naive patients with pSS and age/sex-matched healthy controls. Because of the reported decrease in CD27+ memory B cells in patients [6,40–42], we first quantified peripheral B cells subsets. Proportions of B cells in PBMCs from patients with pSS were higher than in those from healthy controls, possibly due to lymphopenia of CD4+ T cells, as absolute numbers were comparable. In patients with pSS, more circulating B cells were naïve, and proportions of nonswitched and switched memory B cells were decreased (Figure 5A and data not shown).

BTK protein levels were significantly increased in CD27-IgD+ naïve B cells, CD27+IgD- IgM+ nonswitched memory B cells, and CD27+IgD-IgM- switched memory B cells in patients with pSS compared with healthy controls (Figure 5B) (representative flow cytometry dot plots for total B cells are displayed in Supplementary Figure 1). In 16 of 26 patients with pSS (62%), relative BTK expression values in CD27+IgD+ naïve B cells were >1.2-fold those in healthy controls. BTK levels were significantly increased in CD86+ cells within this population, although proportions of CD86+ naïve B cells were similar to those in controls and still very low (0.25% of all naïve B cells) (data not shown). BTK expression correlated with CD86 expression on naïve B cells in patients with pSS and healthy controls (Supplementary Figure 4). Importantly, in patients with pSS, BTK
levels were increased even in naive CD86+ B cells compared to levels in healthy controls. Patients with pSS showed differential BTK expression between different B cell subsets, similar to ACPA-positive RA patients and healthy controls (Supplementary Figure 5). In summary, in a major proportion of patients with pSS, we observed increased BTK protein expression in various B cell subsets, including naive B cells, paralleling our findings in ACPA-positive RA patients.

**FIGURE 4** | Correlation of Bruton's tyrosine kinase (BTK) expression with Th17-lineage cells and inducible costimulator (ICOS) expression on follicular helper T (Tfh) cells. Shown are correlations between BTK protein expression and Th17 cells (CCR6+CXCR3-) (A), Th17.1 cells (CCR6+CXCR3+) (B), Tfh cells (CXCR5+) (C), and ICOS expression by Tfh cells (D), measured by flow cytometry in specimens from ACPA-positive patients (squares) and ACPA-negative patients (triangles). MFI = mean fluorescence intensity.

**BTK expression levels in B cells correlate with serum autoantibodies and parotid gland T cell infiltration in patients with pSS**

Next, we wondered whether BTK levels correlated with clinical or immunologic parameters in patients with pSS. Scores on the EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI) [43] and serum IgG levels were not correlated with relative
BTK levels (data not shown). However, total serum IgM and RF levels correlated significantly with relative BTK levels in total B cells in the 26 patients with pSS (Figures 5C and D). Levels of Ro52- and Ro60-specific antibodies were higher in patients with high BTK expression, although this did not reach significance (data not shown).

**FIGURE 5** | Increased expression of Bruton’s tyrosine kinase (BTK) in B cells from patients with primary Sjogren’s syndrome (pSS). A, Proportions of total B cells among live cells and proportions of naive B cells, IgD⁺ and IgD⁻ nonswitched memory B cells, and switched memory B cells among total B cells in patients with pSS and healthy controls (HC). B, BTK expression measured by intracellular flow cytometry in the indicated B cell subsets in patients with pSS and healthy controls, normalized to BTK expression in total B cells of healthy controls, which was set to 1.0. Data shown are from 6 individual experiments. Representative flow plots show BTK protein expression in B cells from 1 patient with high levels of BTK at baseline, 1 patient with low levels of BTK at baseline, and 1 healthy control. Dashed lines represent the peak of BTK expression in naive B cells from the healthy control. C and D, Correlation of relative BTK levels in B cells with total serum IgM levels (C) and total serum rheumatoid factor (RF) levels (D) in patients with pSS. E, Left, CD3⁺ T cells (brown) in parotid gland biopsy specimens from 2 patients with pSS: 1 with high levels of BTK at baseline and 1 with low levels of BTK at baseline. Original magnification x 40 at top; higher-magnification views are shown at bottom. Right, Correlation of relative BTK expression in total B cells of patients with pSS with CD3⁺ T cell infiltration of the parotid gland. Symbols represent specimens from individual subjects; bars in A and B show the median. MFI = mean fluorescence intensity.
FIGURE 6 | Normalization, upon treatment with abatacept, of Bruton’s tyrosine kinase (BTK) expression levels in B cells from patients with primary Sjogren’s syndrome (pSS). Shown is relative BTK expression in naive B cells (A), IgD⁺ nonswitched memory B cells (B), IgD⁻ nonswitched memory B cells (C), and switched memory B cells (D) from 15 patients with pSS at baseline (time 0 [T0]), upon abatacept treatment (T4, T12, and T24, indicating weeks after initiation of treatment), and after discontinuation of treatment (T36 and T48, indicating weeks after initiation of treatment), normalized to average BTK expression in total B cells from healthy controls (set to 1). Data at left are shown as box plots. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Lines outside the boxes represent minimum and maximum values. Graphs at right show the same patients at T0, T24, and T48. MFI = mean fluorescence intensity.
T cell infiltration in salivary glands is an important feature of pSS. Strikingly, BTK expression levels in total peripheral B cells correlated significantly with numbers of infiltrating CD3\(^+\) T cells/mm\(^2\) in parotid gland infiltrates (Figure 5E). These findings link BTK expression levels to autoantibody levels and salivary gland immune cell infiltration.

**Abatacept treatment normalizes BTK expression in B cells from patients with pSS**

Next, we wanted to investigate whether the increase of BTK expression levels in autoimmunity was dependent on T cell activation. To this end, we examined our 15 patients with pSS who had been treated for 24 weeks with the CTLA-4Ig fusion protein abatacept in the previously reported ASAP study [38], which was aimed at inhibiting T cell activity. Abatacept significantly reduced objective and subjective indicators of disease activity. Scores on the ESSDAI and EULAR Sjögren’s Syndrome Patient Reported Index (ESSPRI) [44] as well as levels of RF and IgG decreased significantly, and health-related quality of life improved during abatacept treatment [38]. Interestingly, abatacept treatment significantly restored BTK expression levels in CD27\(^-\)IgD\(^+\) naïve B cells, CD27\(^+\)IgD\(^+\) and CD27\(^+\)IgD\(^+\)IgM\(^-\) nonswitched memory B cells, and CD27\(^+\)IgD\(^-\)IgM\(^-\) switched memory B cells to levels comparable to those in healthy individuals (Figure 6). Taken together, these findings show that targeting T cell activation in patients with pSS by abatacept restored BTK protein expression in B cells to normal levels after 24 weeks of treatment and suggest the involvement of a B cell- and T cell-driven proinflammatory loop in pSS.

**DISCUSSION**

We previously found in CD19-hBTK-transgenic mice that increased BTK protein levels in B cells are sufficient to disrupt T cell homeostasis and to establish systemic autoimmunity. Therefore, we decided to investigate whether BTK-driven proinflammatory loops may also propagate the development of autoimmune disease in humans. Upon B cell activation, only a small fraction of the BTK molecules present in a cell become detectably phosphorylated in a transient manner. Therefore, in this study, we made use of the unique property of BTK that its protein expression levels are stably up-regulated upon B cell activation [28], allowing for very sensitive measurements of changes in BTK signaling by intracellular flow cytometry. Importantly, BTK protein expression levels correlated with phosphorylation of BTK at Y\(^{551}\). In the present study, we show that BTK expression is up-regulated upon BCR stimulation \textit{in vitro} and that BTK is differentially expressed in human B cell subsets \textit{ex vivo}. Notably, in healthy controls, \textit{ex vivo} BTK expression is significantly increased in CD27\(^+\)IgD\(^+\)IgM\(^+\) and CD27\(^+\)IgD\(^+\)IgM\(^-\)IgD\(^-\) nonswitched memory B cells
compared to CD27\(^{+}\) naïve B cells and CD27\(^{-}\)IgD\(^{+}\)IgM\(^{-}\) switched memory B cells. These findings suggest a role for BTK protein up-regulation in early activation of B cells prior to or during the GC reaction.

BTK protein and phospho-BTK were significantly increased in B cells from ACPA-positive RA patients but not in those from ACPA-negative RA patients. BTK was increased both in naïve and in memory B cells and correlated with frequencies of circulating CCR6\(^{+}\) Th17 cells. As ACPA positivity is associated with a more severe disease course, our findings point towards a pathogenic role of BTK-mediated signaling in RA. Likewise, BTK protein was increased in naïve and memory B cells from a major fraction (~62\%) of patients with pSS and correlated with serum RF levels and parotid gland T cell infiltration. Our data suggest that there were 2 subgroups of patients with pSS, based on BTK expression levels. Although BTK levels correlated with autoantibody levels in serum, they did not show a clear distinction in disease severity (the ESSDAI score) in our cohort of 26 patients. In addition, BTK levels in patients expressing low levels of BTK at baseline decreased upon treatment with abatacept in a manner similar to that in patients expressing high levels of BTK at baseline. Therefore, from the present study, we cannot conclude that these 2 subgroups of patients with pSS represent distinct subsets of patients, except for differences in BTK and autoantibody levels and T cell infiltrates in salivary glands. Analysis of a larger group of patients is needed to draw conclusions about the relationship between BTK expression and disease severity or frequencies of Th17 cells, and about the relationship between different clinical parameters.

We found increased BTK levels in subsets of patients with two distinct autoimmune diseases, indicating that disrupted BTK expression is not a unique feature of one disease but may be involved in more autoimmune diseases. Both RA and pSS are systemic autoimmune diseases, but B cells and autoantibodies have also been implicated in nonsystemic autoimmune diseases. Our data show a clear link between high BTK expression and autoantibodies, suggesting that autoimmune diseases featuring autoantibody production may be interesting candidates for future studies.

Several of our findings point to an association of BTK activity in B cells with T cell activation. These include the observed correlations of BTK expression levels with the frequency of circulating Th17 cells and ICOS expression on Tfh cells in RA patients, and with salivary gland T cell infiltration in pSS. In addition, the specific increase in BTK activity in ACPA-positive RA patients but not in ACPA-negative RA patients may require the involvement of T cells, since ACPA production is very likely T cell-dependent [45–47]. Interestingly, targeting T cell activation in patients with pSS by treatment with abatacept restored BTK protein expression in B cells to normal levels, which suggests that increased BTK expression in circulating B cells of patients with pSS depends on T cell activity. However, it is quite difficult to explain how abatacept treatment would reduce BTK expression in naïve circulating B cells.
A direct effect of abatacept on naive B cells cannot be excluded, since it has been reported that expression of CD86 on B cells, even on naive B cells, is increased in patients with various autoimmune diseases [48,49], and we show in the present study that increased CD86 expression on naive B cells is correlated with higher BTK expression. Nevertheless, our data show that in patients with pSS, BTK expression is even increased in naive resting CD86+ B cells, indicating that elevated BTK levels do not simply reflect the massive B cell activation in these patients. In addition, B cell-T cell interaction through costimulatory molecules [50] and/or T cell-derived cytokines may also affect naive B cells. The observed correlation between BTK levels in peripheral blood B cells and parotid gland T cell infiltration suggests that at an early stage of disease, either T cell activity regulates the expression of BTK in the B cell lineage or elevated BTK levels contribute to local T cell activity. These two scenarios are not mutually exclusive and are supported by our findings in BTK-overexpressing mice, in which the spontaneous autoimmune phenotype is dependent on a B cell-T cell-mediated proinflammatory feedback loop through CD40-CD40L interaction [28,33]. Further experiments are needed to reveal the molecular mechanisms that regulate BTK protein expression in naive B cells in patients with autoimmune disease.

Recent studies have pointed toward a pathogenic role for BTK signaling in autoimmune disease. In B cells from RA patients, BTK signaling was required for induction of interleukin-21 (IL-21) signaling by B cells [51]. Even though levels of phospho-Btk were not significantly different between RF-negative and RF-positive RA patients, there was a significant correlation between phospho-BTK and RF titer. In addition, disease activity in SLE was shown to correlate with expression in peripheral blood B cells of the transcription factor AT-rich-interactive domain-containing protein 3A [52], which interacts directly with BTK [53]. It was recently reported that levels of phospho-Syk, an upstream activator of BTK that has the capacity to phosphorylate BTK at position Y551 [18], were higher in peripheral blood B cells from RA patients, particularly those who were ACPA positive [54]. Those authors also found that treatment with abatacept significantly reduced the levels of phospho-Syk, but it is not known whether phospho-Syk levels are also modulated between different B cell subsets, similar to BTK. In this context, it is remarkable that increased phospho-Syk was found both in treatment-naive RA patients and in patients receiving methotrexate or methotrexate and biologic agents [54]. A recent meta-analysis concluded that the Syk inhibitor fostamatinib has moderate effects in the treatment of RA, with mostly mild-to-moderate adverse events and dose-dependent, transient neutropenia and hypertransaminasemia [55].

In summary, both naive and nonswitched memory B cells in peripheral blood from RA patients and a major fraction of patients with pSS showed significantly increased BTK protein levels, which correlated with ACPA positivity and severity of salivary gland T cell infiltration, respectively. Conversely, in CD19-hBTK-transgenic mice, we have noticed
that BTK overexpression alone is sufficient to disrupt T cell homeostasis and induce Tfh cell formation [28,33]. Furthermore, CD19-hBTK-transgenic mouse B cells show higher production of proinflammatory cytokines IL-6 and interferon-γ, which was dependent on T cells [33]. Together, these findings point to a BTK-dependent proinflammatory feedback loop whereby B cell-T cell interactions through costimulatory molecules and proinflammatory cytokine production drive autoimmunity. Therefore, by interfering with the costimulatory pathways between T cells and CD80/CD86-expressing dendritic cells or activated B cells, abatacept treatment may disrupt this feedback loop. In this context, it is important that it has been shown that strong CD28 engagement to CD86 is crucial for generating the Tfh cells that support GC development [56]. It is therefore attractive to speculate that in autoimmune disease, BTK-mediated signaling in B cells may establish or maintain T cell-propagated pathology and vice versa. Together with the observed beneficial effects of BTK inhibition in mouse models for autoimmune disease and its compelling safety and efficacy in patients with B cell malignancies [18,19], our findings would make BTK an attractive therapeutic target in autoimmune diseases, but we will have to await results of the ongoing clinical trials.

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REFERENCES


## SUPPLEMENTARY MATERIALS

### SUPPLEMENTARY TABLE 1 | Patients characteristics

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<td>52 (37-80)</td>
<td>47 (35-75)</td>
<td>53 (37-82)</td>
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<td>disease duration in months (range)</td>
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<td>6 (1-12)</td>
<td>3 (0-11)</td>
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<td>DAS44 score (range)</td>
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<td>3.0 (1.5-4.6)</td>
<td>2.9 (1.5-4.1)</td>
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<tr>
<td>ACPA in IU/mL (range)</td>
<td>-</td>
<td>454 (14-1802)</td>
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<table>
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<tr>
<th></th>
<th>HC (n=26)</th>
<th>pSS (n=11)</th>
<th>pSS ASAP (n=15)</th>
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<tr>
<td>male / female</td>
<td>4 / 22</td>
<td>0 / 11</td>
<td>3 / 12</td>
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<td>age in years (range)</td>
<td>47 (22-72)</td>
<td>47 (21-70)</td>
<td>43 (18-68)</td>
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<td>disease duration in months (range)</td>
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<td>ESSDAI score (range)</td>
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<td>Anti-Ro52 in AU/mL (range)</td>
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<td>5501 (661-17032)</td>
<td>2580 (32-26430)</td>
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<td>Anti-Ro60 in AU/mL (range)</td>
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<td>7308 (18-29084)</td>
<td>10167 (255-73958)</td>
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Continuous data are presented as median (range). HC = healthy controls, pSS = primary Sjögren's syndrome, RA = rheumatoid arthritis, ASAP = Active Sjögren Abatacept Pilot; DAS44 = Disease Activity Score in 44 joints; ESSDAI = EULAR Sjögren’s Syndrome Disease Activity Index.

### SUPPLEMENTARY TABLE 2

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SUPPLEMENTARY FIGURE 1 | Representative flow plots of total circulating B cells of an ACPA+ and ACPA- RA patient and a healthy control (HC) (A) and a Btk high and BTK low pSS patient and a HC (B). Dotted lines indicate the location of the ACPA+ RA (A) or BTK high pSS (B) B cell population in the flow plots.
SUPPLEMENTARY FIGURE 2 | BTK protein expression in indicated B cell subsets of ACPA+ and ACPA-patients, analyzed by flow cytometry.

SUPPLEMENTARY FIGURE 3 | A, Gating strategy for T cell subsets. B, Representative graph of ICOS expression on naive T cells and Tfh cells.
SUPPLEMENTARY FIGURE 4  |  A, Representative flow plots of CD86- and CD86+ naive B cells (CD19+IgD+CD27-). B, Correlation of BTK protein expression levels with CD86 expression on naive B cells in pSS patients and healthy controls. C, BTK protein expression levels in CD86- and CD86+ naive B cells from pSS patients and healthy controls.

SUPPLEMENTARY FIGURE 5  |  Bruton’s tyrosine kinase (BTK) protein expression levels, as measured by mean fluorescence intensity (MFI) in indicated B cell subsets of 26 pSS patients. Symbols represent individual subjects. Dashed line indicates average MFI in total B cells. *** P value < 0.001.