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

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SUMMARY AND GENERAL DISCUSSION

SUMMARY

Primary Sjögren's syndrome (pSS) is a chronic, systemic autoimmune disease, characterized by lymphocytic infiltration of exocrine glands, and the salivary and lacrimal glands in particular. Predominant symptoms of pSS are a sensation of dry mouth, dry eyes, and chronic fatigue. In addition to the exocrine glands, many other organs can be affected by the disease as well, emphasizing the systemic nature of pSS. Hyperactivity of B lymphocytes is thought to play a central role in the pathogenesis of pSS. Therefore, these cells are considered to be an important target for treatment. More recently, a pathogenic role for T cells has also been recognized, which includes their helper function to (autoreactive) B cells, amongst others. The research described in this thesis addresses two aims: (1) to examine T cell and B cell-related biomarkers of pSS and (2) to assess the effect of rituximab and abatacept treatment on T cell-dependent B cell hyperactivity in pSS patients.

In the first part of this thesis, the relevance of several T cell and B cell-related biomarkers of pSS is described. We evaluated their capacity to predict and/or monitor disease initiation, clinical manifestations, and/or disease progression. In **chapter 2** we reviewed the current knowledge on pathogenicity and plasticity of Th17 cells in pSS. We concluded that Th17 cells/IL-17 producing T cells are involved in local inflammation in pSS, via pro-inflammatory effects on salivary gland epithelial cells and support of ectopic lymphoid tissue formation. The contribution of Th17 cells to systemic disease activity remains, however, more enigmatic. The latter might be a consequence of plasticity of this cell subset. We postulated that plasticity towards Th17.1 cells, co-expressing IL-17 and IFN-γ, may support chronic inflammation and B cell activation in pSS patients.

In addition to Th17 cells, a more dedicated subset of B cell helper T cells, named T follicular helper (Tfh) cells, has been identified. In **chapter 3a** and **3b** we described studies assessing the prevalence and phenotype of circulating Tfh (cTfh) cells and their regulatory counterparts, i.e. T follicular regulatory (Tfr) cells, in pSS patients and controls. We showed that frequencies of both subsets were increased in blood from pSS patients compared to healthy controls. Circulating Tfr (cTfr) cells were even further increased than cTfh cells, resulting in significantly higher cTfr/cTfh ratios in pSS patients, compared with either healthy controls (**chapter 3a**) or non-SS sicca controls (**chapter 3b**). Frequencies of cTfh cells and cTfr cells correlated with serum levels of IgG and CXCL13, and with systemic disease activity, as measured by the EULAR Sjögren's syndrome disease activity index (ESSDAI) and clinical ESSDAI. These results indicate that cTfh and cTfr cell frequencies are useful biomarkers of systemic disease activity in pSS. The positive correlation between cTfr cell frequencies, B cell hyperactivity and systemic disease activity is, however, remarkable, as Tfr cells are supposed to suppress humoral immune responses. A possible explanation for a reduced suppressive capacity of Tfr

cells in pSS comes from our finding that circulating Treg cells, and in particular cTfr cells, from pSS patients express decreased levels of the inhibitory receptor CTLA-4.

The next two chapters focused on B cell-related biomarkers of pSS. In the study described in **chapter 4** we aimed to characterize a subset of epithelium-associated B cells expressing Fc-receptor-like protein 4 (FcRL4). The presence of FcRL4$^+$ B cells around and within the ductal epithelium of inflamed glandular tissue can be seen as a histologic hallmark of pSS. Furthermore, these cells are possibly precursor cells of mucosa-associated lymphoid tissue (MALT) lymphoma, a type of B cell lymphoma that occurs in 5-10% of pSS patients, preferentially in the parotid glands. Because FcRL4 is widely expressed by MALT lymphomas, the presence of large numbers of non-neoplastic FcRL4$^+$ B cells in parotid gland tissue may identify patients who are at risk of lymphoma development. For the purpose of characterization, we isolated 'normal' FcRL4$^+$ B cells from parotid gland tissue of pSS patients without MALT lymphoma and performed single cell RNA sequencing. We found that FcRL4$^+$ B cells from parotid glands of pSS patients showed upregulation of genes involved in homing and cell adhesion, consistent with their tissue location close to the epithelium. FcRL4$^+$ B cells also showed upregulation of genes that promote inflammation and B cell survival. We postulated that these cells contribute significantly to the epithelial damage seen in the glandular tissue of pSS patients, and that these cells are prone to lymphomagenesis.

Following up on identification of potential biomarkers of MALT lymphoma, we described in **chapter 5** that 50% of pSS patients with salivary gland MALT lymphoma had an aberrant ratio of serum immunoglobulin free light chains (FLC), with a relative increase in FLCκ compared to FLCλ. In pSS patients without MALT lymphoma, levels of both FLCκ and FLCλ were often increased, but abnormal ratios were rarely seen. We concluded that the FLC κ/λ ratio is a useful biomarker of MALT lymphoma presence, which can be used in combination with conventional biomarkers such as cryoglobulinemia, lymphopenia, low complement levels, and persistent parotid gland enlargement. In this chapter we also showed that serum levels of FLCκ, and to a lesser extent FLCλ, can be used to monitor the effect of immunomodulatory treatment on B cell activity in pSS patients.

In the second part of this thesis we assessed the effect of rituximab and abatacept treatment on T cell-dependent B cell hyperactivity in pSS patients. In **chapter 6** we described a study in which we showed that B cell depletion therapy with rituximab had significant effects on the T cell compartment, in addition to the well-described effects on the B cell compartment. Among T cells, in particular cTfh cells were affected and frequencies of these cells were normalized to levels seen in healthy controls. The reduction in cTfh cells was associated with improved objective clinical disease activity measures. In **chapter 7** we summarized and discussed current literature on clinical

and biological effects of rituximab treatment in pSS. We concluded that rituximab has beneficial effects on B cell activity, glandular morphology, dryness, fatigue and several extraglandular manifestations in at least subgroups of pSS patients. Available evidence suggested that patients with moderate to severe systemic involvement, i.e. activity in multiple ESSDAI domains, may benefit most from treatment. In addition to B cell-targeting therapies, abatacept treatment (aiming at inhibition of T cell activation) also showed beneficial clinical effects in pSS patients. In **chapter 8** we described the effect of this treatment on T cell homeostasis and T cell-dependent B cell hyperactivity in pSS. Abatacept treatment reduced numbers of cTfh cells, as well as expression of the activation marker ICOS on T cells, both in the periphery and locally in parotid gland tissue. The decrease in ICOS expression on the remaining cTfh cells was significantly associated with the reduction in ESSDAI scores over time. B cell hyperactivity was also decreased by abatacept treatment, as reflected by lower levels of circulating plasmablasts and autoantibody titers.

Finally, we showed in the study described in **chapter 9** that abatacept treatment resulted in decreased expression levels of Bruton's tyrosine kinase (BTK) in B cells from pSS patients. BTK is a signaling molecule that directly links B cell receptor (BCR) signals to B cell proliferation and survival. At baseline, BTK protein expression was increased in a majority of pSS patients, and correlated with serum rheumatoid factor levels and parotid gland T cell infiltration. Together with the findings described in **chapter 8**, these observations illustrate the pivotal role of the crosstalk between B cells and T(fh) cells in the pathogenesis of pSS.

GENERAL DISUSSION

To date, treatment options for primary Sjögren's syndrome (pSS) are still symptomatic. Although several immunomodulatory treatments show promising clinical and biological outcomes, heterogeneity in clinical signs and immune activation patterns between patients hampers successful drug development and registration. To address this issue, biomarkers that enable stratification of clinical and molecular phenotypes and identification of new, (patient-)specific, targets for treatment are urgently needed. In this thesis we aimed to evaluate new biomarkers and treatment targets by elucidating the role of T cell-dependent B cell hyperactivity in the pathogenesis of pSS. This chapter discusses the key findings of this thesis and identifies areas for future research.

Part I. T cell-dependent B cell hyperactivity: Biomarker of disease?

After the Th1/Th2 paradigm for adaptive immunity was challenged, the role of newly recognized effector subsets, including Th17 cells and T follicular helper (Tfh) cells, gained much attention. In the past decade, these new subsets have been extensively studied, in particular in the context of inflammatory diseases and autoimmunity [1,2]. $CD4^+$ effector cell subsets can be discriminated based on chemokine receptor expression patterns and/or cytokine producing capabilities. However, gradually it became clear that $CD4^+$ T cell effector subsets are not necessarily committed to a single differentiation fate, but that certain subsets show plasticity, i.e. the ability to adapt different effector functions [3]. For example, $CD4^+$ T cells that co-express IL-17 and IFN-γ (named Th17.1 cells) have been associated with chronic inflammation [4]. Tfh cells also come in different phenotypes, and can be sub-divided in Tfh1, Tfh2, and Tfh17 cells, based on expression patterns of CXCR3 (associated with Th1 cells) and CCR6 (associated with Th17 cells) [5].

Th17 cells in pSS

Th17 cells and their signature cytokine IL-17 are present in inflamed salivary glands of pSS patients [6–8]. In the glands they may contribute to the disease by activation of epithelial cells and stimulation of ectopic lymphoid tissue formation (reviewed in **chapter 2**). The current literature demonstrates that Th17 cells play a crucial role in initiation and progression of disease in several mouse models of SS [9–11]. However, the contribution of Th17 cells to human pSS in ambiguous, which may be a result of significant plasticity as well as phenotypic heterogeneity of this cell subset. To complicate things further, the definition of Th17 cells by either cytokine production or chemokine receptor expression is still a matter of debate. We proposed in **chapter 2** that local differentiation of Th17 cells towards Th17.1 cells, co-expressing IL-17 and IFN-γ, contributes to chronic inflammation and B cell activation in the inflamed glands.

However, addressing the fate and functionalities of infiltrated T cells in the inflamed exocrine glands of pSS patients remains a challenge, because of limited availability of fresh biopsy material, especially at different time points of disease development, and changes in environmental cues when cells are isolated for *in vitro* fate-mapping and functional studies.

Tfh cells and T follicular regulatory cells in pSS

Although the necessity of T cell help for antibody responses was described decades ago, the recognition of a dedicated subset of B cell helper T cells, named Tfh cells, followed much later. First, the chemokine receptor CXCR5, promoting migration to B cell follicles, was linked to Tfh cells [12]. Other key molecules such as BCL6, IL-21, PD-1 and ICOS were subsequently revealed. However, no single marker or combination of markers can unequivocally identify Tfh cells, as expression of Tfh cell-related molecules is dynamic and heterogeneous [2]. Tfh cells facilitate B cell activation, and increased numbers of Tfh cells have been associated with several B cell-mediated autoimmune diseases, including pSS [13]. We found in several study cohorts that frequencies of circulating Tfh cells, defined as $CD4^+CD45RA^-CXCR5^+PD\text{-}1^+$ cells, were increased in pSS patients compared with healthy individuals (see **chapters 3a, 6 & 8**). This increase is already present at the time of diagnosis, and Tfh cell frequencies correlated with serum IgG levels and systemic disease activity scores, as measured by EULAR Sjögren's syndrome disease activity index (ESSDAI) and clinical ESSDAI (clinESSDAI: ESSDAI without the biological domain[14]). Circulating Tfh cells are therefore a useful biomarker of B cell hyperactivity and systemic disease activity in pSS, and can be used to monitor extraglandular involvement in pSS patients over time.

Recently, a regulatory subset of Tfh cells, named T follicular regulatory (Tfr) cells, has been identified [15]. These cells are able to control Tfh cell proliferation and consequently B cell activation (reviewed by [16]). **Chapters 3a** and **3b** of this thesis described the frequency and phenotype of circulating Tfr (cTfr) cells in a large group of pSS patients. In addition to cTfh cells, frequencies of cTfr cells were elevated in pSS patients compared with healthy individuals. Furthermore, increased expression of the chemokine receptor CXCR3 was observed on cTfh and cTfr cells from pSS patients. Expression of CXCR3 enables migration from the circulation towards inflamed glandular tissues where CXCL10, an important ligand for CXCR3, is produced [17]. The importance of Tfr cells for regulation of antigen-specific immune responses was recently illustrated in a *Bcl6$^{fl/fl}$Foxp3Cre/Cre* mouse model, in which Tfr cells were diminished [18]. When this mouse model was combined with an experimental Sjögren's syndrome (ESS) model, in which mice are immunized with salivary gland proteins, disease started earlier and worsened. Tfr-deficient mice showed enhanced serum levels of autoantibodies against

salivary gland proteins and increased frequencies of germinal center (GC) B cells in the cervical lymph nodes. On the other hand, when a knock-out model for Tfh cells (*Bcl6$^{fl/fl}$Cd4Cre*) was combined with the ESS model, mice were protected from lymphocytic infiltration, excessive GC responses and autoantibody production, while salivary flow was not greatly improved [18]. Together, these results underline the importance of Tfr/Tfh cell balance in protecting mice from autoimmune disease. However, these cells do not seem to contribute significantly to the impairment of saliva production in the ESS model. Similarly, the relation between local immune responses and hyposalivation in human pSS remains unclear, and there is a relatively weak association between saliva production and the degree of salivary gland inflammation [19]. While a lack of Tfr cells can exacerbate autoimmune disease in mice, we showed in **chapters 3a** and **3b** that cTfr cells in pSS patients were even further increased than cTfh cells, resulting in a significantly higher cTfr/cTfh ratio. An increased Tfr/Tfh ratio in pSS patients was also recently described by Fonseca et al. [20,21]. Of note, in their studies Tfr cells and Tfh cells were defined by expression of CXCR5 only. We included CXCR5^{+}PD-1^{+} cells, because the gene expression profile of circulating PD-1^{+} memory Tfh is more polarized towards Tfh cells, and these cells exhibit a greater B helper capacity compared to CXCR5^{+}PD-1^{-} cells [22].

Whereas an expanded regulatory cell population would suggest increased immune suppression, this is clearly not the case in pSS. We reported in **chapter 3a** that cTfr cell frequencies correlate positively with serological markers of B cell activity and systemic disease activity. Interestingly, measurement of CTLA-4 expression in cTfr cells showed that levels of this inhibitory receptor are significantly lower in pSS patients, compared with healthy individuals. CTLA-4 is a critical receptor that mediates suppression of humoral immune responses by regulatory T cells [23,24]. The importance of this receptor in immune homeostasis is illustrated by the finding that CTLA-4-deficient mice die from T cell-dependent multi-organ infiltration [25,26]. Mutations in the *CTLA4* gene in humans that result in haploinsufficiency were associated with a complex dominant immune dysregulation syndrome, with clinical features that are related to autoimmunity (e.g., cytopenia) as well as immunodeficiency (e.g., recurrent infections) [27]. Decreased expression of CTLA-4 by Tfr cells in pSS may -at least partially- explain why control of Tfh cell expansion and B cell responses in pSS are impaired (Figure 1). Our data reinforce the need for additional functional studies to assess suppressive capacity by regulatory T cells in this disease.

Epithelium-associated B cells in pSS

A characteristic histopathological finding in salivary gland lesions of pSS patients is infiltration of B cells, located in close proximity to, or even within, the ductal epithelium. A substantial proportion of the intra-epithelial B cells express Fc receptor-like protein

4 (FcRL4) [28], and these cells seem to contribute significantly to the formation of lymphoepithelial lesions (LELs). FcRL4 is an inhibitory receptor that can bind IgA and is typically expressed by B cells residing in mucosa-associated lymphoid tissue (MALT) [29,30]. Binding of IgA possibly functions as a negative feedback mechanism to control formation of IgA-producing plasma cells. Furthermore, FcRL4$^+$ B cells in the salivary glands of pSS patients may serve as precursor cells of salivary gland MALT lymphoma, as FcRL4 is widely expressed by these lymphomas [29]. To investigate if the presence of FcRL4$^+$ B cells can act as a biomarker to identify patients at risk of MALT lymphoma development, additional knowledge on the origin, function and fate of FcRL4$^+$ B cells is necessary.

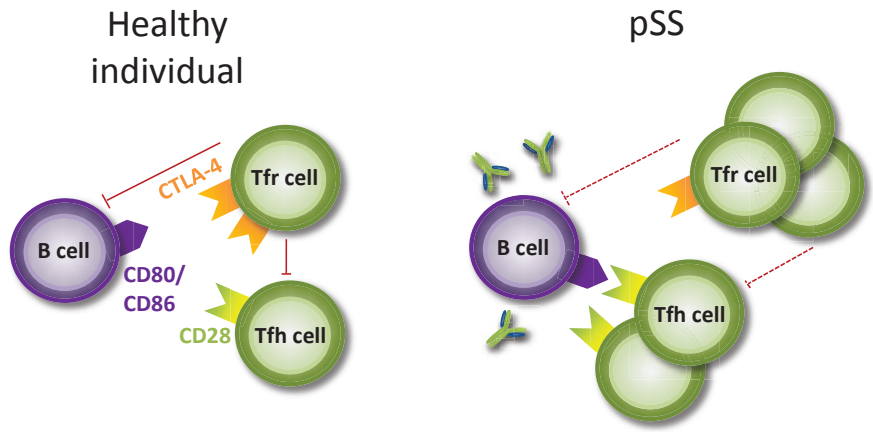

FIGURE 1 | Reduced immune suppression by T follicular regulatory cells in patients with primary Sjögren's syndrome. In healthy individuals, T follicular regulatory (Tfr) cells suppress activation of B cells and T follicular helper (Tfh) cells through CTLA-4. In pSS patients, frequencies of circulating Tfr cells are increased, while expression levels of CTLA-4 by these cells are decreased. Consequently, suppression of B cell responses as well as Tfh cell proliferation by Tfr cells in pSS patients may be impaired. CTLA-4: cytotoxic T-lymphocyte–associated antigen 4.

In **chapter 4** the prevalence and phenotype of circulating FcRL4$^+$ B cells in pSS patients and non-SS sicca patients was assessed to explore the possibility that these cells are increasingly activated at mucosal tissue sites and then migrate, via the blood, to the inflamed salivary glands. We observed, however, no difference in the frequency of circulating FcRL4$^+$ B cells between pSS patients and non-SS sicca patients, and the prevalence was generally low (±0.5% of B cells). An alternative possibility could be that FcRL4 is locally upregulated by infiltrated B cells upon stimulation by epithelial cells, T cells or other environmental triggers. This hypothesis is supported by the finding that stimulation of 'healthy' human memory B cells with CD40L and a TLR9-agonist induces FcRL4 expression by the majority of memory B cells [31].

To characterize local FcRL4$^+$ B cells in pSS, these cells were isolated from parotid gland tissue of patients and single-cell RNA sequencing was performed. Sequencing results revealed that the transcriptional profile of FcRL4$^+$ B cells is more similar to FcRL4$^-$CD27$^+$ 'memory' cells, than FcRL4$^-$CD27$^-$ 'naive' cells. Consistent with the phenotype that we observed in blood, increased transcript levels of *CXCR3* were found in glandular FcRL4$^+$ B cells. As mentioned before, expression of CXCR3 may facilitate migration to inflamed salivary gland tissue, and specifically to the ductal epithelium, where its ligand CXCL10 is produced. Additionally, compared with FcRL4-negative B cells, FcRL4$^+$ B cells showed significant upregulation of integrins, the NFκB-pathway and pro-survival factors including the BAFF/APRIL receptor TACI. The upregulation of integrins, including CD11c, by FcRL4$^+$ B cells may explain their retention within the epithelium. Upregulation of the NFκB-pathway could be a result of increased ligation of TACI by BAFF and/or APRIL [32]. Ligation of TACI can also result in AID induction, and consequently isotype switching or somatic hypermutation [33]. In line with this notion, sequencing data from single cells revealed that transcript levels of AID were enriched in FcRL4$^+$ B cells. Of note, histopathological analysis of the corresponding diagnostic biopsies of the included patients showed that the infiltrates did not harbor GCs, based on the H&E staining. Together, the results described in **chapter 4** suggest that glandular FcRL4$^+$ B cells in pSS are chronically activated, pro-inflammatory B cells, which may undergo isotype switching and/or somatic hypermutation at extrafollicular sites. The expression of AID, together with a high proliferation rate and expression of pro-survival factors by FcRL4$^+$ B cells in salivary gland tissues of pSS patients may put these cells at risk of mutagenesis. A genetic predisposition, for example a polymorphism of *TNFAIP3* (A20: a protein that inhibits NFκB signaling) [34], could be an additional risk factor. As a consequence, pSS patients who harbor FcRL4$^+$ B cells in their salivary glands may be at risk of MALT lymphoma development. The finding that more FcRL4$^+$ B cells are present in parotid glands, compared to labial glands, may explain why MALT lymphomas in pSS patients preferentially develop in parotid glands [28]. Although histology is the gold standard to confirm a diagnosis of MALT lymphoma, taking a biopsy is invasive and progression cannot be monitored easily over time. Serological markers of MALT lymphoma may therefore aid in 1) identifying patients at risk, and 2) monitoring disease progression and response to treatment in daily clinical practice.

Serological markers of B cell hyperactivity

The central role of B cell hyperactivity in pSS pathogenesis is widely recognized. In addition to conventional biomarkers of B cell activity such as serum levels of total IgG and autoantibodies, several other B cell-related markers have been investigated in serum of pSS patients. These include β2-microglobulin, BAFF, CXCL13 and immunoglobulin

free light chains (FLC) [35–37]. In **chapter 5**, serum levels of FLC in pSS and non-SS sicca patients are presented. FLC levels, and in particular FLCκ, correlated with systemic disease activity, as measured by ESSDAI as well as clinESSDAI. We further showed that the FLC κ/λ ratio is a potential biomarker of salivary gland MALT lymphoma, as 50% of the MALT lymphoma patients had an aberrant ratio. A recent study identified cryoglobulinemia, parotid gland enlargement and lymphadenopathy as strong predictors of MALT lymphoma presence in pSS [38]. However, in a previous study we reported that only 11% (4/35) of MALT lymphoma patients had cryoglobulinemia, indicating that this biomarker lacks sensitivity [39]. On the other hand, 77% (27/35) of MALT-pSS patients experienced parotid gland swelling [39], but this symptom is also frequent in pSS patients without lymphoma [40]. Our study showed that the κ/λ ratio might serve as a valuable additional biomarker to identify and monitor patients with MALT lymphoma. A larger, prospective study is needed to prove its predictive value in addition to previously recognized predictive factors, such as cryoglobulinemia and persistent parotid gland enlargement. In **chapter 5** we also presented longitudinal data of FLC levels in pSS patients before and after treatment with either rituximab or abatacept. The data reported in this chapter indicate that FLC levels are useful for monitoring the effect of treatment on B cell activity, because the FLC levels have a shorter half-life than IgG and are sensitive to change.

Part II. T cell-dependent B cell hyperactivity: Target for treatment?

Effects of rituximab treatment on T cell-dependent B cell hyperactivity

B cell depletion therapy with rituximab (anti-CD20) was one of the first biologic disease-modifying anti-rheumatic drugs (DMARD) that was clinically tested in pSS. Up to now, several studies have evaluated the efficacy of rituximab in pSS, with inconsistent outcomes (reviewed in **chapter 7**). To understand variability in clinical response between pSS patients, it is important to study the effects of treatment on the immune system. In **chapter 6** we assessed the effects of rituximab treatment on the T cell compartment of pSS patients. We hypothesized that depletion of B cells, and consequently inhibition of antigen presentation and cytokine production by these cells, would affect T cell activation. Indeed, numbers and frequencies of cTfh cells were significantly decreased during B cell depletion, and to a lesser extent also circulating Th17 cells were reduced. In addition, serum levels of IL-21 and IL-17 were significantly lowered by treatment. Importantly, the decrease in cTfh cells correlated with the decrease in ESSDAI scores during B cell depletion. Numbers and frequencies of Th1 cells and Th2 cells were unaffected by treatment.

The specific effects observed on cTfh cells and Th17 cells can be explained by lower availability of IL-6 due to the depletion of B cells. This cytokine is involved in

the differentiation and activation of Tfh cells as well as Th17 cells [41]. Plasmablasts in particular produce high amounts of IL-6 [42]. A decrease in serum levels of IL-6 was indeed observed in a previous study [43]. Together, these results underline the importance of the IL-6/IL-21 axis in pSS pathogenesis. In addition to the observed effects on the T cell compartment, several other beneficial biologic effects of rituximab treatment in pSS have been shown (for a review see **chapter 7**). These effects include (partial) restoration of salivary gland morphology and reduction of autoantibody levels [44,45]. The restorative effects on salivary gland morphology involves the reduction in number and severity of the LELs, which seems to be a direct consequence of the depletion of $FcRL4^+$ B cells located within the epithelium [44]. These findings also illustrate the crosstalk between the $FcRL4^+$ B cells and the epithelium.

Effects of abatacept treatment on T cell-dependent B cell hyperactivity

Because $CD4^+$ T cells and B cells seem to act in a pro-inflammatory feedback loop in pSS patients, therapies that impair T cell activation are also feasible treatment options. Abatacept, a fully human fusion molecule combining cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) with IgG Fc, can bind to CD80/86 on antigen presenting cells (APC) and hereby inhibits T cell activation by these APCs. In **chapter 8** we studied the effects of abatacept treatment on T cells and B cells from treated pSS patients. We showed that specifically cTfh cells and peripheral Treg (pTreg) cells are reduced by treatment, and that the remaining cTfh cells express lower levels of inducible costimulator (ICOS), which is usually upregulated upon activation. The decrease in ICOS expression by cTfh cells was significantly associated with the decrease in ESSDAI scores during treatment. Importantly, protein levels of ICOS were also locally reduced in the inflamed salivary glands after 24 weeks of treatment. We did not specifically assess frequencies of cTfr cells in this study, but the decrease in cTfh cells as well as pTreg cells, which comprise cTfr cells, suggests that these cells are also reduced by abatacept treatment.

In addition to the observed effects on cTfh cells, pTreg cells, and ICOS expression, we showed that frequencies of circulating plasmablasts and serum levels of anti-SSA/Ro and anti-SSB/La were significantly decreased during treatment, which is likely a result of impaired differentiation of memory B cells into plasmablasts and short-lived plasma cells. The effects on cTfh cells and B cell activity were further reflected by the observed decrease in the number of GCs in parotid glands of treated patients [46]. Despite the observed effects on systemic and local B cell activity, total numbers of infiltrated T cells and B cells, and protein expression levels of IL-21 in parotid gland tissue, were not significantly affected by abatacept (**chapter 8** and [46]). Apparently, migration of lymphocytes into the inflamed tissue was not impaired, and IL-21 production was maintained.

Whether the number of Tfh cells in the glandular tissue is affected by abatacept treatment still needs to be investigated. However, also other $CD4^+$ T cell subsets may contribute to the production of IL-21 [47,48], and these cells may be activated via CD40-CD40L-mediated interaction with APCs, and/or as a result of continuous presence of IL-6 in the glands. Another explanation for continued local IL-21 production after abatacept treatment may come from a study in rheumatoid arthritis patients, showing that a subset of CD28-negative $CD4^+$ memory T cells infiltrated synovial tissues and maintained pro-inflammatory cytokine production [49]. Downregulation of CD28 by $CD4^+$ T cells may also occur in salivary gland tissue of pSS patients, and could influence the effectivity of costimulation blockade [50]. A third explanation for continued IL-21 production might be that mainly new formation of effector T cells is inhibited by abatacept and that turnover of the residing memory cells is relatively slow. A repeated biopsy after a longer period of treatment (e.g., one year) would be needed to address this issue. Lastly, we cannot exclude that drug penetration of salivary gland tissue is suboptimal compared to other tissues (e.g., synovial tissue and secondary lymphoid organs). Differences in drug penetration between tissues may be an additional explanation for the modest effects of abatacept treatment on salivary gland inflammation, while this drug can ameliorate multiple extraglandular manifestations of pSS.

The role of Bruton's tyrosine kinase in T-cell dependent B cell hyperactivity in pSS

Bruton's tyrosine kinase (BTK) is a key molecule involved in B cell receptor (BCR) signaling. In mice, overexpression of BTK in B cells results in a Sjögren/lupus-like autoimmune phenotype upon ageing, in a T cell-dependent manner [51,52]. Whether aberrant BTK levels were also involved in human autoimmunity was unclear. In **chapter 9** we showed that intracellular levels of BTK in B cells are increased in pSS patients and ACPA-positive rheumatoid arthritis patients. Although the highest increase was observed in memory B cells, also naive B cells from these patients showed increased BTK expression compared with healthy controls, indicating that this increase is not merely a result of chronic antigen exposure. In pSS patients, increased expression levels of BTK were associated with higher levels of RF and with higher numbers of infiltrated T cells in the parotid glands. The association with RF levels may be explained by a lower threshold for B cell activation when BTK expression, and consequently BCR signaling, is enhanced, resulting in enhanced plasma cell formation. A greater antigen-presenting potential by B cells as a result of higher BTK expression levels may explain the observed association with numbers of infiltrated T cells [53].

Interestingly, BTK expression levels in both naive and memory B cells were significantly decreased during abatacept treatment. This decrease could be a direct effect of abatacept on B cells via binding to CD80/86, as increased expression levels of CD86 on

naive B cells were associated with higher BTK expression. In addition, altered levels of T cell-derived cytokines during abatacept treatment may affect B cell activation and possibly also BTK expression levels, although such molecular mechanisms are relatively unexplored.

FUTURE PERSPECTIVES

Patient stratification by immune profiling

Because pSS is a heterogeneous disease, patient stratification could aid in the development of patient-tailored treatments. Potential biomarkers described in this thesis that can be used as a starting point for patient stratification are frequencies of Tfh cells and Tfr cells in blood, and possibly also BTK expression levels. Others have shown that the presence of an interferon (IFN) type I signature identifies a subgroup of pSS patients with high systemic disease activity and high levels of autoantibodies [54,55]. Whether the IFN signature represents a different pathologic mechanism, or whether it only indicates more active disease, remains to be shown [56].

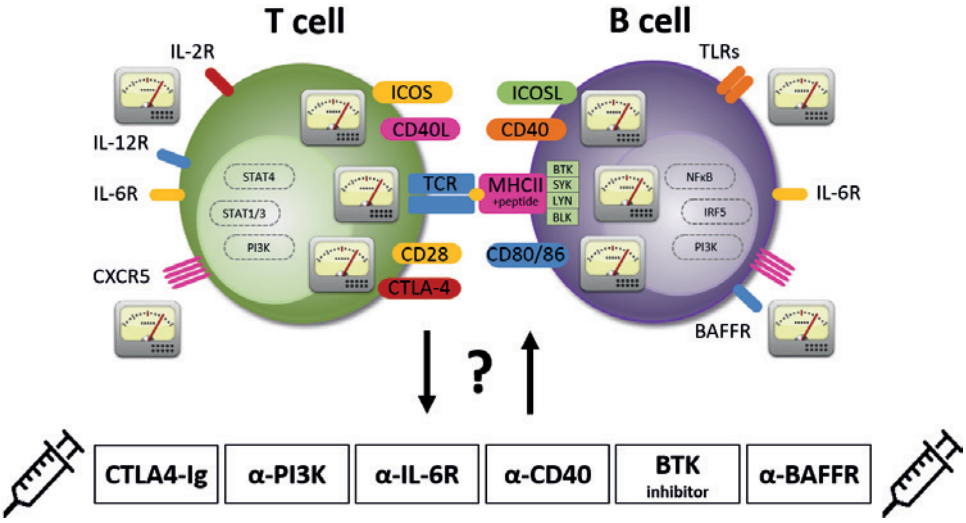

FIGURE 2 | Matching of patient-specific immune signaling and treatment. Critical immune signals, the sum of which determines T or B cell activation and expansion, are illustrated. Measuring the strength of each signal in an individual may aid in the establishment of patient-tailored treatment. Drugs that are currently under investigation in pSS target costimulatory pathways (CTLA4-Ig, α-CD40), cytokine signaling (α-IL-6R, α-BAFFR) or intracellular signaling (α-PI3K, BTK inhibitor).

So far, neither results from immunophenotyping studies nor genome wide association studies (GWAS) have facilitated patient stratification in pSS, and other approaches to identify patient-specific immune signals that contribute to pathogenesis

are needed. The majority of the currently available research, including the research presented in this thesis, assessed specific immune signals in large numbers of cells simultaneously and often on a patient group level. However, recent technologic advances such as (single cell) whole transcriptome sequencing have enabled us to measure an almost infinite number of immune signals at the same time. The amount of data generated by these techniques asks for a network-based approach. A potential way forward is to measure signaling differences in T cells and B cells from pSS patients on a single-cell and single-patient level (Figure 2). Such an approach enables detection of small, intrinsic changes in T cell and B cell signaling that are probably present in pSS patients, but still need to be unraveled. Subsequently, patient-specific immune profiles can be determined and personalized treatment with targeted biologicals can be established.

Promising treatments that target T cell-dependent B cell hyperactivity

Multiple biologic treatments that interfere with T cell-B cell interaction are currently under investigation in pSS. B cell-targeting therapies are still considered as potential treatment strategies, and the beneficial biologic effects that are seen after treatment of pSS patients with rituximab support further development of these therapies. In addition to rituximab, promising drug candidates that result in (partial) B cell depletion are epratuzumab (anti-CD22) and VAY736 (anti-BAFFR). The addition of epratuzumab to standard therapy in SLE patients did not result in higher response rates compared with placebo [57]. However, in a subgroup of anti-SSA-positive SLE patients with associated SS, response rates were higher in patients who received epratuzumab, compared to placebo [58]. Interestingly, these SLE patients with associated SS showed a faster and stronger B cell depletion compared to SLE patients without associated SS. Treatment with anti-BAFFR also resulted in B cell depletion [59], and clinical efficacy of this treatment in pSS is currently under investigation. An advantage of targeting BAFF-R is that in addition to B cell depletion, BAFF-BAFF-R signaling in the remaining B cells (i.e. plasmablasts and plasma cells) is inhibited. Intriguingly, while it is a non-depleting antibody, anti-CD40 treatment also showed promising effects in pSS patients by reducing ESSDAI scores significantly [60]. The main mechanism of action of anti-CD40 is probably inhibition of T cell-dependent B cell activation. However, CD40 can also be expressed by other cell types, including dendritic cells (DCs), and anti-CD40 may therefore exert additional B cell-independent effects, such as inhibition of T cell activation by DCs.

Other promising treatments for pSS that affect T cell-dependent B cell hyperactivity, without depletion of B cells, are abatacept (CTLA-4Ig), tocilizumab (anti-IL-6R), JAK1 inhibitors (e.g., filgotinib), and PI3Kδ inhibitors. Interestingly, these drug candidates may affect the formation of Tfh cells, as we have shown for abatacept. IL-6 is important for

differentiation of these cells, and JAK1 and PI3Kδ are involved in downstream signaling of IL-6R [61]. Lastly, BTK inhibitors are potential drug candidates to interfere with T cell-dependent B cell activation in pSS, in particular in patients with high BTK expression levels at baseline. Because BTK expression levels were associated with the amount of T cell infiltration in the inflamed glands of pSS patients, and also with frequencies of circulating Th17 cells in ACPA$^+$ RA patients, BTK inhibition may affect both T cells and B cells in the periphery and the affected tissues.

CONCLUDING REMARKS

Although no disease-modifying treatments for pSS patients exist to date, the understanding of the pathogenesis of pSS has increased significantly over the last decade. The use of new therapeutic options has contributed significantly to this increase in knowledge. Many promising therapies are currently under investigation and at least one of these agents tested will probably be approved in a not-too-distant future. We showed that Tfh cells are a useful biomarker and treatment target for pSS. We presume that interruption of T cell-B cell interaction, at either side, is crucial for successful treatment of systemic disease activity in this disease. The contribution of lymphocytic infiltration to exocrine gland dysfunction is, however, still poorly understood and needs further investigation. A challenge for the future is to treat patients as early as possible to prevent damage to the exocrine glands, which is apparently irreversible once initiated. Another challenge for future research is to unravel patient heterogeneity, possibly by the detection of clinical and molecular disease phenotypes, to enable personalized treatment of pSS patients.

REFERENCES

1 Patel DD, Kuchroo VK. Th17 Cell Pathway in Human Immunity: Lessons from Genetics and Therapeutic Interventions. *Immunity* 2015;**43**:1040–51.

2 Vinuesa CG, Linterman MA, Yu D, *et al*. Follicular Helper T Cells. *Annu Rev Immunol* 2016;**34**:335–68.

3 DuPage M, Bluestone JA. Harnessing the plasticity of CD4+ T cells to treat immune-mediated disease. *Nat Rev Immunol* 2016;**16**:149–63.

4 Ramesh R, Kozhaya L, McKevitt K, *et al.* Pro-inflammatory human Th17 cells selectively express P-glycoprotein and are refractory to glucocorticoids. *J Exp Med* 2014;**211**:89–104.

5 Schmitt N, Bentebibel SE, Ueno H. Phenotype and functions of memory Tfh cells in human blood. *Trends Immunol* 2014;**35**:436–42.

6 Nguyen CQ, Hu MH, Li Y, *et al.* Salivary gland tissue expression of interleukin-23 and interleukin-17 in Sjögren's syndrome: findings in humans and mice. *Arthritis Rheum* 2008;**58**:734–43.

7 Sakai a., Sugawara Y, Kuroishi T, *et al.* Identification of IL-18 and Th17 cells in salivary glands of patients with Sjogren's syndrome, and amplification of IL-17-mediated secretion of inflammatory cytokines from salivary gland cells by IL-18. *J Immunol (Baltimore, Md 1950)* 2008;**181**:2898–906.

8 Katsifis GE, Rekka S, Moutsopoulos NM, *et al.* Systemic and Local Interleukin-17 and Linked Cytokines Associated with Sjögren's Syndrome Immunopathogenesis. *Am J Pathol* 2009;**175**:1167–77.

9 Lin X, Rui K, Deng J, *et al.* Th17 cells play a critical role in the development of experimental Sjögren's syndrome. *Ann Rheum Dis* 2015;**74**:1302–10.

10 Voigt A, Esfandiary L, Wanchoo A, *et al.* Sexual dimorphic function of IL-17 in salivary gland dysfunction of the C57BL/6.NOD-Aec1Aec2 model of Sjögren's syndrome. *Sci Rep* 2016;**6**:38717.

11 Iizuka M, Tsuboi H, Matsuo N, *et al.* A crucial role of RORγt in the development of spontaneous Sialadenitis-like Sjögren's syndrome. *J Immunol* 2015;**194**:56–67.

12 Bryant VL, Ma CS, Avery DT, *et al*. Cytokine-mediated regulation of human B cell differentiation into Ig-secreting cells: predominant role of IL-21 produced by CXCR5+ T follicular helper cells. *J Immunol* 2007;**179**:8180–90.

13 Simpson N, Gatenby PA, Wilson A, *et al.* Expansion of circulating T cells resembling follicular helper T cells is a fixed phenotype that identifies a subset of severe systemic lupus erythematosus. *Arthritis Rheum* 2010;**62**:234–44.

14 Seror R, Meiners P, Baron G, *et al.* Development of the ClinESSDAI: a clinical score without biological domain. A tool for biological studies. *Ann Rheum Dis* 2016;**75**:1945–50.

15 Linterman MA, Pierson W, Lee SK, *et al.* Foxp3+ follicular regulatory T cells control the germinal center response. *Nat Med* 2011;**17**:975–82.

16 Sage PT, Sharpe AH. T follicular regulatory cells in the regulation of B cell responses. *Trends Immunol* 2015;**36**:410–8.

17 Ogawa N, Ping L, Zhenjun L, *et al.* Involvement of the interferon-gamma-induced T cell-attracting chemokines, interferon-gamma-inducible 10-kd protein (CXCL10) and monokine induced by interferon-gamma (CXCL9), in the salivary gland lesions of patients with Sjögren's syndrome. *Arthritis Rheum* 2002;**46**:2730–41.

18 Fu W, Liu X, Lin X, *et al.* Deficiency in T follicular regulatory cells promotes autoimmunity. *J Exp Med* 2018;:jem.20170901.

19 Daniels TE, Cox D, Shiboski CH, *et al.* Associations between salivary gland histopathologic diagnoses and phenotypic features of Sjögren's syndrome among 1,726 registry participants. *Arthritis Rheum* 2011;**63**:2021–30.

20 Fonseca VR, Romão VC, Agua-Doce A, *et al.* Blood T Follicular Regulatory Cells / T Follicular Helper Cells ratio Marks Ectopic Lymphoid Structure Formation and PD-1 $^{+}$ ICOS $^{+}$ T Follicular Helper Cells Indicate Disease Activity in Primary Sjögren's Syndrome. *Arthritis Rheumatol* Published Online First: 23 January 2018.

21 Fonseca VR, Agua-Doce A, Maceiras AR, *et al.* Human blood T_{fr} cells are indicators of ongoing humoral activity not fully licensed with suppressive function. *Sci Immunol* 2017;**2**:eaan1487.

22 Locci M, Havenar-Daughton C, Landais E, *et al.* Human circulating PD-1+CXCR3-CXCR5+ memory Tfh cells are highly functional and correlate with broadly neutralizing HIV antibody responses. *Immunity* 2013;**39**:758–69.

23 Wing JB, Ise W, Kurosaki T, *et al.* Regulatory T cells control antigen-specific expansion of Tfh cell number and humoral immune responses via the coreceptor CTLA-4. *Immunity* 2014;**41**:1013–25.

24 Sage PT, Paterson AM, Lovitch SB, *et al.* The coinhibitory receptor CTLA-4 controls B cell responses by modulating T follicular helper, T follicular regulatory, and T regulatory cells. *Immunity* 2014;**41**:1026–39.

25 Tivol EA, Borriello F, Schweitzer AN, *et al.* Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity* 1995;**3**:541–7.

26 Waterhouse P, Penninger JM, Timms E, *et al.* Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. *Science* 1995;**270**:985–8.

27 Schubert D, Bode C, Kenefeck R, *et al.* Autosomal dominant immune dysregulation syndrome in humans with CTLA4 mutations. *Nat Med* 2014;**20**:1410–6.

28 Haacke EA, Bootsma H, Spijkervet FKL, *et al.* FcRL4+ B-cells in salivary glands of primary Sjögren's syndrome patients. *J Autoimmun* 2017;**81**:90–8.

29 Falini B, Agostinelli C, Bigerna B, *et al.* IRTA1 is selectively expressed in nodal and extranodal marginal zone lymphomas. *Histopathology* 2012;**61**:930–41.

30 Wilson TJ, Fuchs A, Colonna M. Cutting edge: human FcRL4 and FcRL5 are receptors for IgA and IgG. *J Immunol* 2012;**188**:4741–5.

31 Jourdan M, Robert N, Cren M, *et al.* Characterization of human FCRL4-positive B cells. *PLoS One* 2017;**12**:e0179793.

32 Gardam S, Brink R. Non-Canonical NF-κB Signaling Initiated by BAFF Influences B Cell Biology at Multiple Junctures. *Front Immunol* 2014;**4**:509.

33 Castigli E, Wilson SA, Scott S, *et al.* TACI and BAFF-R mediate isotype switching in B cells. *J Exp Med* 2005;**201**:35–9.

34 Nocturne G, Tarn J, Boudaoud S, *et al.* Germline variation of TNFAIP3 in primary Sjögren's syndrome-associated lymphoma: Table 1. *Ann Rheum Dis* 2016;**75**:780–3.

35 Gottenberg JE, Seror R, Miceli-Richard C, *et al.* Serum levels of beta2-microglobulin and free light chains of immunoglobulins are associated with systemic disease activity in primary Sjogren's syndrome. Data at enrollment in the prospective ASSESS cohort. *PLoS One* 2013;**8**:e59868.

36 Nocturne G, Seror R, Fogel O, *et al.* CXCL13 and CCL11 Serum Levels and Lymphoma and Disease Activity in Primary Sjögren's Syndrome. *Arthritis Rheumatol (Hoboken, NJ)* 2015;**67**:3226–33.

37 Pollard RP, Abdulahad WH, Vissink A, *et al.* Serum levels of BAFF, but not APRIL, are increased after rituximab treatment in patients with primary Sjogren's syndrome: data from a placebo-controlled clinical trial. *Ann Rheum Dis* 2013;**72**:146–8.

38 Brito-Zerón P, Kostov B, Fraile G, *et al.* Characterization and risk estimate of cancer in patients with primary Sjögren syndrome. *J Hematol Oncol* 2017;**10**:90.

39 Pollard RP, Pijpe J, Bootsma H, *et al.* Treatment of mucosa-associated lymphoid tissue lymphoma in Sjogren's syndrome: a retrospective clinical study. *J Rheumatol* 2011;**38**:2198–208.

40 Ramos-Casals M, Brito-Zerón P, Solans R, *et al.* Systemic involvement in primary Sjogren's syndrome evaluated by the EULAR-SS disease activity index: analysis of 921 Spanish patients (GEAS-SS Registry). *Rheumatology (Oxford)* 2014;**53**:321–31.

41 Hunter CA, Jones SA. IL-6 as a keystone cytokine in health and disease. *Nat Immunol* 2015;**16**:448–57.

42 Chavele KM, Merry E, Ehrenstein MR. Cutting edge: circulating plasmablasts induce the differentiation of human T follicular helper cells via IL-6 production. *J Immunol (Baltimore, Md 1950)* 2015;**194**:2482–5.

43 Pollard RP, Abdulahad WH, Bootsma H, *et al.* Predominantly proinflammatory cytokines decrease after B cell depletion therapy in patients with primary Sjogren's syndrome. *Ann Rheum Dis* 2013;**72**:2048–50.

44 Delli K, Haacke EA, Kroese FGM, *et al.* Towards personalised treatment in primary Sjögren's syndrome: baseline parotid histopathology predicts responsiveness to rituximab treatment. *Ann Rheum Dis* 2016;**75**:1933–8.

45 Verstappen GM, Kroese FGM, Meiners PM, *et al.* B cell depletion therapy normalizes circulating follicular TH cells in primary Sjögren syndrome. *J Rheumatol* 2017;**44**:49–58.

46 Haacke EA, van der Vegt B, Meiners PM, *et al.* Abatacept treatment of patients with primary Sjögren's syndrome results in a decrease of germinal centres in salivary gland tissue. *Clin Exp Rheumatol* 2017;**35**:317–20.

47 Wei L, Laurence A, Elias KM, *et al.* IL-21 is produced by Th17 cells and drives IL-17 production in a STAT3-dependent manner. *J Biol Chem* 2007;**282**:34605–10.

48 Rao DA, Gurish MF, Marshall JL, *et al.* Pathologically expanded peripheral T helper cell subset drives B cells in rheumatoid arthritis. *Nature* 2017;**542**:110–4.

49 Warrington KJ, Takemura S, Goronzy JJ, *et al.* CD4+,CD28- T cells in rheumatoid arthritis patients combine features of the innate and adaptive immune systems. *Arthritis Rheum* 2001;**44**:13–20.

50 Xu H, Perez SD, Cheeseman J, *et al.* The allo- and viral-specific immunosuppressive effect of belatacept, but not tacrolimus, attenuates with progressive T cell maturation. *Am J Transplant* 2014;**14**:319–32.

51 Kil LP, de Bruijn MJW, van Nimwegen M, *et al.* Btk levels set the threshold for B-cell activation and negative selection of autoreactive B cells in mice. *Blood* 2012;**119**:3744–56.

52 Corneth OBJ, de Bruijn MJW, Rip J, *et al.* Enhanced Expression of Bruton's Tyrosine Kinase in B Cells Drives Systemic Autoimmunity by Disrupting T Cell Homeostasis. *J Immunol* 2016;**197**:58–67.

53 Sharma S, Orlowski G, Song W. Btk regulates B cell receptor-mediated antigen processing and presentation by controlling actin cytoskeleton dynamics in B cells. *J Immunol* 2009;**182**:329–39.

54 Brkic Z, Maria NI, van Helden-Meeuwsen CG, *et al*. Prevalence of interferon type I signature in CD14 monocytes of patients with Sjogren's syndrome and association with disease activity and BAFF gene expression. *Ann Rheum Dis* 2013;**72**:728–35.

55 Maria NI, Brkic Z, Waris M, *et al*. MxA as a clinically applicable biomarker for identifying systemic interferon type I in primary Sjogren's syndrome. *Ann Rheum Dis* 2014;**73**:1052–9.

56 Kroese FGM, Verstappen GM, De Leeuw K, *et al*. Sjögren's syndrome, should we sign? *Expert Rev Clin Immunol* 2016;**12**.

57 Clowse MEB, Wallace DJ, Furie RA, *et al*. Efficacy and Safety of Epratuzumab in Moderately to Severely Active Systemic Lupus Erythematosus: Results From Two Phase III Randomized, Double-Blind, Placebo-Controlled Trials. *Arthritis Rheumatol* 2017;**69**:362–75.

58 Gottenberg J-E, Dörner T, Bootsma H, *et al*. Efficacy of Epratuzumab, an Anti-CD22 Monoclonal IgG Antibody, in Systemic Lupus Erythematosus Patients with Associated Sjögren's Syndrome: *Post-hoc* Analyses from the EMBODY Trials. *Arthritis Rheumatol* Published Online First: 30 January 2018.

59 Doerner T, Posch M, Wagner F, *et al*. Safety and Efficacy of Single Dose VAY736 (anti-BAFF-R mAb) in Patients with Primary Sjögren's Syndrome (pSS) - ACR Meeting Abstracts. In: *Arthritis Rheumatol. 2016; 68 (suppl 10)*.

60 Fisher B, Zeher M, Ng WF, Bombardieri M, *et al*. The Novel Anti-CD40 Monoclonal Antibody CFZ533 Shows Beneficial Effects in Patients with Primary Sjögren's Syndrome: A Phase IIa Double-Blind, Placebo-Controlled Randomized Trial - ACR Meeting Abstracts. In: *Arthritis Rheumatol. 2017; 69 (suppl 10)*.

61 Calabrese LH, Rose-John S. IL-6 biology: implications for clinical targeting in rheumatic disease. *Nat Rev Rheumatol* 2014;**10**:720–7.