Local gene therapy and the identification of therapeutic targets in Sjögren’s syndrome
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THE EXPRESSION OF APRIL IN SJÖGREN’S SYNDROME: ABERRANT EXPRESSION OF APRIL IN THE SALIVARY GLAND

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ABSTRACT

Objective: A proliferation inducing ligand (APRIL) and B cell-activating factor (BAFF) are B cell-related mediators and may play a role in the pathogenesis in Sjögren’s syndrome. In this descriptive study we assessed the expression of APRIL and BAFF in the minor salivary gland and serum from Sjögren’s syndrome (SS) patients.

Methods: Paraffin-embedded minor salivary gland sections from SS, sicca controls and healthy volunteers were analyzed by immunohistochemistry. Digital image quantification was performed to evaluate the expression of BAFF, APRIL, and their receptors. Furthermore, serum was analyzed for soluble BAFF and APRIL levels by enzyme-linked immunosorbent assay. All the data were also separated based on decreased and normal stimulated flow and analyzed independent of their classification.

Results: APRIL expression was lower in minor salivary gland biopsies from SS patients compared with healthy volunteers and to a lesser extend sicca controls, whereas BAFF expression was similar in both groups. Soluble APRIL levels in serum were increased in autoantibody positive SS patients and in subjects with decreased salivary flow independent of the classification.

Conclusions: APRIL salivary gland tissue levels are decreased, suggesting that targeting this cytokine locally in the salivary glands would not benefit SS patients. Moreover, the discrepancy between local and systemic levels is striking and future research should assess this in more detail.
INTRODUCTION

Sjögren’s syndrome (SS) is a systemic autoimmune disorder characterized by lymphocytic infiltration in the exocrine glands and the recruitment of activated and memory B cells in the salivary gland infiltrates. A proliferation inducing-ligand (APRIL) and B cell-activating factor of the tumor necrosis factor family (BAFF) have powerful roles in B cell biology. They are involved in augmentation of B cell antigen presentation, co-stimulation of B cell activation, enhancement of B cell survival, regulation of B cell tolerance and germinal center maintenance. These ligands share two tumor necrosis factor (TNF) family member receptors, transmembrane activator and CAML interactor (TACI) and B cell maturation antigen (BCMA). In addition, BAFF binds BAFF-receptor (BAFF-R) and APRIL binds heparin sulphate proteoglycans (HSPGs). Unlike BAFF, APRIL does not exist in a membrane-bound form, although a cell surface fusion protein with TWEAK is described as TWE-PRIL.

Recent studies have localized APRIL to immune cell subsets that also produce BAFF; monocytes, macrophages, dendritic cells, and T cells. Moreover, stimulation of these cells with interferon (IFN)γ and IFNα, results in upregulation of APRIL and BAFF mRNA. Additionally, non-immune cells can express APRIL, including osteoclasts and tumor tissues. BAFF is also expressed by cells outside the immune system, such as fibroblast-like synoviocytes in the synovium of patients with rheumatoid arthritis (RA) and salivary gland epithelial cells in both patients with SS and healthy individuals. IFNα and IFNγ induce BAFF mRNA and protein secretion in salivary gland epithelial cells and the human salivary gland cell line, with a higher increase in salivary gland epithelial cells derived from SS patients compared with controls. BAFF induction is independent of interleukin-10 (IL-10) and TNF alone. This has never been described for APRIL.

In SS, increased BAFF has been reported in serum and salivary glands, and correlates with disease parameters. Similarly, increased serum APRIL levels correlate with focus score and serum IgG. Recently, APRIL production in the salivary gland was found to be low in SS patients. In this study, sicca controls were not investigated and no associations with other systemic and local symptoms were made. The reduced or absent expression of APRIL in the salivary glands from SS patients is surprising since it is thought to enhance autoimmune disease by sustained B cell activation. Other data suggesting a complicated role for APRIL in autoimmune diseases include the description of an inverse association between circulating APRIL levels and BAFF levels and APRIL levels and other disease parameters in systemic lupus erythematosus (SLE) patients.

Although the expression level of APRIL in SS compared to sicca controls, which did not fulfill the European-American (EA) criteria for primary SS (pSS), and healthy volunteers and the correlation with systemic features or salivary flow is largely unknown, APRIL has been brought up as a therapeutic target for SS. Therefore, we studied the expression of APRIL, BAFF and their receptors in minor salivary gland biopsies and APRIL and BAFF in serum from SS patients, sicca controls and healthy volunteers and correlated these levels with the salivary flow.
MATERIAL AND METHODS

Patients and controls
All 17 patients included in this study fulfilled the European-American (EA) criteria for primary SS (pSS) and were recruited from the Sjögren’s syndrome clinic at the National Institutes of Dental and Cranial Research (NIDCR), National Institutes of Health (NIH), Bethesda, MD, USA. The control group consisted of 5 gender and age-matched healthy volunteers (HV) and 9 gender and age-matched patients evaluated for sicca symptoms not meeting the European-American (EA) criteria for pSS (N; Table 1). All subjects signed an informed consent and the study was approved by the Institutional Review Board (IRB) of the National Institutes of Dental and Craniofacial Research (NIDCR).

Laboratory assays
Data on serum autoantibodies, immunoglobulins (IgG, IgM, IgA), erythrocyte sedimentation rate (ESR), minor salivary gland focus score, and stimulated salivary flow were obtained by NIDCR’s Sjögren’s clinic staff and evaluated as part of the routine diagnostic evaluation for SS. A focus score is defined as a number lymphocytic foci, which are adjacent and normal-appearing mucus acini and contain more than 50 lymphocytes, per 4mm² of glandular tissue. BAFF and APRIL levels in serum were determined using a commercial ELISA kit (R&D systems, Minneapolis, MN, USA) according to the manufacturer’s protocol. Before BAFF detection, serum samples were pre-treated with protein-A sepharose (Sigma-Aldrich, St. Louis, MO, USA) to prevent possible interference with rheumatoid factor immunoglobulin.

Immunohistochemistry and digital quantification
Paraffin sections of minor salivary gland biopsies were stained after heat-induced citrate antigen retrieval with the following antibodies: mouse anti-human APRIL (Aprily-2, Alexis Biochemicals, San Diego, CA, USA), rabbit anti-human BCMA (ProSci Incorporation, Poway, CA, USA), mouse anti-human TACI (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-human BAFF-R (Alexis Biochemicals), and rabbit anti-Perlecan (H-300, Santa Cruz Biotechnology). For each staining, isotype controls were included; mouse IgG1 (Dako, Carpinteria, CA, USA), rat IgM (BD biosciences, San Jose, CA, USA), and rabbit IgG (R&D systems). The secondary antibodies goat anti-mouse HRP (Dako), goat anti-rat HRP (Southern Biotechnology, Birmingham, AL, USA), and goat anti-rabbit HRP (Dako) were used. Staining was developed with AEC substrate (Vector Laboratories, Burlingame, CA, USA). All sections were randomly analyzed by computer-assisted image analysis using 400x magnification. The images of the high-power fields were analyzed both within and outside infiltrates, using the Qwin analysis system (Leica, Cambridge, UK), as described previously. Positive staining for the cellular markers was expressed as the number of positive cells/mm² and the staining for the cytokine markers as integrated optical density (IOD)/mm².

Statistical analysis
Differences in immunohistochemistry, serum APRIL and serum BAFF between experimental groups (>2 groups) were assessed using the Kruskal-Wallis test followed
Table 1. Clinical, laboratory and histological characteristics of study subjects.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>HV (n=5)</th>
<th>N (n=9)</th>
<th>SS (n=17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr), mean (sd)</td>
<td>53.0 (12.0)</td>
<td>46.8 (12.2)</td>
<td>53.4 (15.0)</td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>5/5 (100.0)</td>
<td>9/9 (100.0)</td>
<td>16/17 (94.1)</td>
</tr>
<tr>
<td>ANA positive, n (%)</td>
<td>1/5 (20.0)</td>
<td>3/9 (33.3)</td>
<td>13/16 (82.3)</td>
</tr>
<tr>
<td>ENA positive, n (%)</td>
<td>0/5 (0.0)</td>
<td>0/9 (0.0)</td>
<td>11/16 (68.8)</td>
</tr>
<tr>
<td>SSA/Ro positive, n (%)</td>
<td>0/5 (0.0)</td>
<td>0/9 (0.0)</td>
<td>8/17 (47.1)</td>
</tr>
<tr>
<td>SSB/La positive, n (%)</td>
<td>0/5 (0.0)</td>
<td>0/9 (0.0)</td>
<td>10/17 (58.8)</td>
</tr>
<tr>
<td>SSA+SSB positive, n (%)</td>
<td>0/5 (0.0)</td>
<td>0/9 (0.0)</td>
<td>8/17 (47.1)</td>
</tr>
<tr>
<td>ESR, mean (sd)</td>
<td>17 (11)</td>
<td>29 (27)</td>
<td></td>
</tr>
<tr>
<td>IgG (mg/dL), mean (sd)</td>
<td>971 (188)</td>
<td>1447 (669)</td>
<td></td>
</tr>
<tr>
<td>IgA (mg/dL), mean (sd)</td>
<td>165 (99)</td>
<td>331 (155)</td>
<td></td>
</tr>
<tr>
<td>IgM (mg/dL), mean (sd)</td>
<td>108 (41)</td>
<td>156 (90)</td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydroxychloroquine, n (%)</td>
<td>4/9 (44.4)</td>
<td>2/17 (11.8)</td>
<td></td>
</tr>
<tr>
<td>Dose</td>
<td>1/17: 200 mg/day</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3/9: 400 mg/day -</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1/9: 600 mg/day 1/17: 600 mg/day</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oral steroids, n (%)</td>
<td>2/9 (22.2)</td>
<td>2/17 (11.8)</td>
<td></td>
</tr>
<tr>
<td>Dose</td>
<td>-</td>
<td>1/17: 5 mg/day</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1/9: 10 mg/day -</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>1/9: 40 mg/day -</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>1/17: 60 mg/day</td>
<td></td>
</tr>
<tr>
<td>Methotrexate, n (%)</td>
<td>1/9 (11.1)</td>
<td>0/17 (0.0)</td>
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<tr>
<td>Dose</td>
<td>10 mg/week</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>MALT lymphoma, n (%)</td>
<td>0/5 (0.0)</td>
<td>0/9 (0.0)</td>
<td>1/15 (6.7)</td>
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<tr>
<td>Focus score, mean (sd)</td>
<td>1.00 (0.45)</td>
<td>0.44 (0.88)</td>
<td>4.24 (3.47)</td>
</tr>
<tr>
<td>EGM, n (%)</td>
<td>0/5 (0.0)</td>
<td>0/9 (0.0)</td>
<td>5/17 (29.4)</td>
</tr>
<tr>
<td>Vasculitis</td>
<td>1/17 (5.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peripheral neuropathy</td>
<td>2/17 (11.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arthritis</td>
<td>2/17 (11.8)</td>
<td></td>
<td></td>
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</tbody>
</table>

ANA: antinuclear antibodies; ENA: antibodies to extractable nuclear antigens; EGM: extra glandular manifestation; ESR: erythrocyte sedimentation rate; SSA: antibodies to Ro antigens; SSB: antibodies to La antigens; SS: patients fulfilling the European-American (EA) criteria for primary Sjögren’s syndrome (pSS); HV: healthy volunteers (n=5); N: patients evaluated for sicca symptoms not meeting the EA criteria for pSS (n=9).
by the non-parametric Mann Whitney test. The p-values were not adjusted for multiple comparisons. All analyses were performed with GraphPad Prism statistical software (GraphPad Software Inc. version 5.01, La Jolla, CA, USA) using a p-value ≤ 0.05 as statistically significant.

RESULTS
Decreased APRIL and TACI expression in the SGs from SS patients
Minor salivary gland sections from healthy volunteers (HV), sicca controls (N) and SS patients were analyzed using immunohistochemistry and quantitative digital image analysis to locate and quantify the expression of APRIL, BAFF and their receptors, TACI and BCMA, respectively. In each subject from each group, eighteen high-power fields with foci and eighteen high-power fields without foci were analyzed and averaged to randomize the expression of the markers for localization within the salivary gland. APRIL, BAFF, TACI and BCMA were expressed in the salivary duct cells of each group (Figure 1 shows representative pictures). In SS patients, BAFF and BCMA showed a different

Figure 1. APRIL, BAFF, TACI and BCMA expression in the minor salivary glands. Immunohistochemical analysis of APRIL, BAFF, TACI and BCMA expression (red staining) in minor salivary gland biopsies from healthy volunteers (HV), sicca controls (N) and SS patients. For each antibody staining (in red) the isotype control and representative examples are shown. Original magnification: 400x. For reference, arrows indicate ductal epithelium, triangles indicate acinar cells and asterisks indicate focal infiltrates.
staining pattern when compared with APRIL and TACI, with more pronounced BAFF and BCMA staining in ductal cells and inflammatory foci (Figure 1). Quantification of the immunohistochemical staining revealed decreased APRIL expression in the minor salivary glands of SS patients compared with healthy volunteers (18123 IOD/mm² and 41283 IOD/mm² respectively, p = 0.02), and the expression in sicca patients was similar to those with healthy volunteers (p = 0.52; Figure 2A). In contrast, BAFF expression was similar between both SS (320655 IOD/mm²) and healthy volunteers (411685 IOD/mm², p = 0.35), and SS and sicca controls (269056 IOD/mm², p = 0.36; Figure 2A). TACI expression, like APRIL, was decreased in SS compared with healthy volunteers (16.0 counts/mm² and 158.0 counts/mm² respectively, p = 0.02), but not in sicca controls (29.0 counts/mm², p = 0.55). Although numerically more individuals with sicca complaints had lower TACI levels compared with healthy volunteers, no statistical significance was reached (p = 0.08; Figure 2B). The other shared receptor for

**Figure 2.** Decreased APRIL and TACI expression is correlated in ductal cells from SS minor salivary glands. Quantification of APRIL, BAFF (A), TACI, and BCMA (B) expression using digital image analysis. Soluble cytokines (APRIL and BAFF) are expressed as integrated optical density (IOD)/mm² and receptors (TACI and BCMA) are given as positive cell count/mm². The horizontal bar is the median value and the p-values were determined by the Kruskal-Wallis test followed by the non-parametric Mann Whitney test and significant differences were indicated (*). HV = healthy volunteers, N = sicca controls, SS = Sjögren’s syndrome patients.
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APRIL and BAFF, BCMA, was not differentially expressed in SS patients compared with healthy volunteers \( (p = 0.48) \). Interestingly, the expression of BCMA was significantly different for SS compared with sicca controls (2035 counts/mm\(^2\) and 813 counts/mm\(^2\) respectively, \( p = 0.02; \) Figure 2B). Heparan sulfate proteoglycans (HSPG, Perlecan) on the cell surface have recently been described as interactors with APRIL\(^{23}\), but staining for these molecules did not show any difference for the 3 groups (data not shown).

Increased APRIL serum levels in anti-Ro and -La positive SS patients

APRIL and BAFF are elevated in serum in other autoimmune diseases, e.g. RA and SLE\(^{24}\). Therefore, we measured APRIL and BAFF levels in the serum from SS patients. SS patients showed higher systemic levels of APRIL compared with healthy volunteers (3.95 ng/ml versus 1.83 ng/ml; \( p = 0.02 \)) and sicca controls (2.16 ng/ml; \( p = 0.03 \); Figure 3A). In 41% (7 out of 17) of the SS patients, APRIL was detectable above baseline levels, and sub-analysis of the SS group showed that 71% (5 out of 7) of the patients with positive autoantibodies against Ro/SSA and La/SSB had high (> 20 ng/ml) APRIL levels (Figure 3B). APRIL expression was not different between SS patients with and without extra glandular manifestations (EGM, see table 1). Serum BAFF levels in SS patients

![Figure 3](image_url)

Figure 3. Increased APRIL serum levels in anti-SSA/SSB positive SS patients. Serum from healthy volunteers (HV), sicca controls (N), and Sjögren's syndrome patients (SS) were analyzed for APRIL (A) and BAFF (C) levels using ELISA. In the SS group, the serum APRIL levels were subdivided for the presence of autoantibodies (B) against SSA and SSB. The horizontal bar is the median value and the p-values were determined by the Kruskal-Wallis test followed by the non-parametric Mann Whitney test and significant differences were indicated (*). Data on the y-axis for figure B is plotted in log-scale. SSA = Sjögren’s syndrome A/Ro, SSB = Sjögren’s syndrome B/La.
(759 pg/ml) were not significantly different when compared with healthy volunteers (743 pg/ml, p = 0.59), or with sicca controls (545 pg/ml, p = 0.12; Figure 3C).

Patients with decreased salivary flow have increased serum APRIL and TACI expression in the salivary glands

One of the hallmarks of SS is decreased salivary flow. Therefore, data from all the markers in the salivary gland and serum were analyzed for decreased and normal stimulated salivary flow, using 1.5ml/15min as a cutoff, independent of the classification. Serum APRIL and salivary gland TACI levels were significantly increased in subjects with decreased salivary flow (Figure 4 A and B respectively, both p = 0.02). For all the other markers, no significant changes were found.

DISCUSSION

APRIL has been shown to be up-regulated in RA and SLE24. Recently, APRIL expression in the salivary gland of SS patients was found to be low compared with healthy controls. In that study, sicca controls were not tested and APRIL levels were not correlated with systemic features or salivary flow16. It is known that epithelial salivary gland cells of SS patients play a role in the presentation of antigens25, and are potent producers and secretors of BAFF11. In contrast, the expression level and role of APRIL in epithelial cells is less well known. Therefore, we have setup a descriptive study for the APRIL expression in minor salivary gland biopsies and serum of SS patients, sicca controls and healthy volunteers. In addition, we assessed the expression of the receptors for BAFF and APRIL. We found by immunohistochemical analysis of minor salivary gland...
tissue that APRIL was expressed in the ductal epithelium of all individuals. Interestingly, minor salivary gland biopsies of SS patients showed decreased APRIL expression compared with control groups. Systemically, APRIL levels were high in 41% of the patients and all these patients were double positive for Ro and La autoantibodies. Furthermore, APRIL serum levels correlated with reduced salivary flow independent of the classification criteria.

In contrast to BAFF expression, minor salivary gland biopsies showed APRIL expression mainly in the ductal epithelial cells, and not in the inflammatory foci. Moreover, the overall APRIL expression was decreased in SS patients compared with healthy volunteers. Previously, gene expression profiling of minor salivary glands by microarray, showed lower APRIL expression in SS patients, but this change was not significant26. Our data are in line with a recent report, showing no up-regulation of APRIL in Sjögren’s sialadenitis lesions16. In contrast to the expression in salivary glands, APRIL levels in serum were elevated in SS patients, especially in anti-Ro/La positive patients. The discrepancy between serum and salivary gland levels of APRIL raises the question where APRIL is produced. Additional research will have to answer this question, but based on our data and on a previous study16, it is unlikely that this comes from the SG, since immunohistochemistry showed that APRIL is primarily present inside the epithelial cells and not in the inflammatory foci.

Interestingly, serum APRIL levels and TACI expression in the salivary glands were also increased in subjects with decreased salivary flow, independent from the diagnosis of SS. The relationship of systemic APRIL and salivary gland dysfunction is puzzling and more research on the function of APRIL in SS needs to be done to elucidate any possible connection. One may postulate that the association with TACI and salivary gland dysfunction is based on increased BAFF binding to TACI in an inflammatory environment in SS that results in internalization of the receptor or may mask the receptor from antibody binding in IHC. Further studies are needed to also evaluate this relationship.

We showed the presence of the receptors TACI and BCMA in the salivary glands of SS patients, sicca controls, and healthy volunteers. Staining of these receptors was primarily seen in epithelial duct cells. In addition, BCMA was also detected in the infiltrating cells. Although these receptors are known for their expression on lymphocytes, BAFF/APRIL receptors are also detected on non-immune cells. Expression of BAFF-R and TACI, for instance, has been observed in mammary epithelial cells and normal thyroid gland27,28. Adipocytes also express BAFF and APRIL, together with their receptors, and are thought to act in an auto/paracrine way for regulation and differentiation of these cells29. It is possible that a similar regulatory mechanism takes places in the salivary gland epithelial cells. Further research is necessary to confirm this finding. TACI expression in minor salivary gland biopsies from SS patients was decreased compared with healthy volunteers. In contrast, BCMA expression was up-regulated in SS minor salivary gland compared with sicca controls, but showed no difference when compared with healthy volunteers. Previously, microarray studies showed increased BCMA gene expression in SS minor salivary glands compared with
sicca controls\textsuperscript{26}. The different expression pattern suggests a different function for these receptors in the salivary gland.

Previous studies have shown overexpression of BAFF in lymphocytes in minor salivary glands from SS patients and equal expression in epithelial cells compared with healthy volunteers\textsuperscript{11,13,30}. Our data showed expression of BAFF in lymphocytic infiltrates in SS as well as duct epithelial cells of both healthy volunteers and SS patients. However, we did not detect an overall difference in the expression of BAFF in SS minor salivary glands compared with our control group. It is possible that the total BAFF expression did not change, when infiltrates and epithelium were assessed. In addition, the similar levels may be due to intrinsic limitations of immunohistochemistry, which is a very informative method to detect overall expression levels in tissue, but does not distinguish the specific cell types expressing the protein of interest. In addition, it may be that BAFF is only transiently expressed in patients. This is supported by a previous study showing that mRNA levels of BAFF are similar in SS and control salivary epithelial cells. Only ex vivo stimulation with TNF\textsubscript{α} and IFN\textsubscript{γ} resulted in increased BAFF mRNA expression in SS patients compared with sicca controls\textsuperscript{11}.

We showed no difference in serum BAFF levels between SS patients and controls. Literature shows conflicting results on the BAFF levels in sera from SS patients; some find higher levels in patients, while others do not detect differences\textsuperscript{31,32}. A reason for these opposing results may be that BAFF fluctuates with changes in disease activity\textsuperscript{33}. Technical limitations of the chosen assay, interference with rheumatoid factor and the stability of the BAFF protein may also explain the differences. Recently, a new ELISA has been developed for detection of both glycosylated and nonglycosylated BAFF, which should not be affected by rheumatoid factor. This ELISA may lead to more consistent results\textsuperscript{34}.

In conclusion, APRIL levels were low in the minor salivary glands of SS patients whereas high serum levels were found compared to sicca controls and healthy volunteers. This suggests that compared to BAFF, APRIL may have a less pronounced pro-inflammatory role in minor salivary gland pathogenesis in SS patients. Moreover, it is tempting to suggest that APRIL has anti-inflammatory effects in the minor salivary glands, and that SS patients may therefore benefit more from BAFF blockade alone, instead of targeting both APRIL and BAFF, but more research needs to explore this hypothesis.

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