Human gingival fibroblasts are a source of B cell-activating factor during periodontal inflammation

Ahed Dyab | Ava Emnegard | Magnus Wänman | Filippa Sjöström | Elin Kindstedt

Abstract

**Background:** Host-modulating therapy is a possible treatment for individuals that respond poorly to conventional periodontal therapy. B cells, abundant in periodontitis lesions, require the cytokines B cell-activating factor (BAFF) and A proliferation-inducing ligand (APRIL) for survival and maturation. Although mRNA levels of BAFF and APRIL are increased in tissue from periodontitis lesions, it is unknown if periodontal resident cells express BAFF and/or APRIL during periodontal inflammation. In this study, we aim to analyze the expression of BAFF and APRIL in human gingival fibroblasts after stimulation with proinflammatory cytokines. Furthermore, we perform protein analysis in tissues and serum from periodontitis patients and healthy controls.

**Methods:** Human gingival fibroblasts were cultured and stimulated with the proinflammatory cytokines’ tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β). The mRNA expression of BAFF and APRIL was analyzed by real-time quantitative polymerase chain reaction (qPCR), and the protein was detected in tissue sections using immune staining. Serum levels of BAFF were analyzed with enzyme-linked immunosorbent assay (ELISA).

**Results:** In gingival fibroblasts, TNF-α upregulated BAFF mRNA, but APRIL was unaffected. IL-1β affected neither BAFF nor APRIL expression. BAFF protein was detected in the oral epithelium and in cells of the underlying connective tissue in periodontitis tissue, and BAFF protein was increased in the serum of periodontitis patients.

**Conclusion:** Periodontal resident cells express BAFF during periodontal inflammation and participate in providing a favorable milieu for the survival and action of B cells.

**Keywords**
A proliferation-inducing ligand protein, B cell-activating factor, fibroblasts, inflammation, periodontitis
1 | INTRODUCTION

In periodontitis, tooth-supporting tissues are progressively and irreversibly destroyed due to an imbalanced immune response. The sequelae of periodontitis, tooth loss and impaired oral function, are associated with social disability and reduced self-experienced quality of life. Furthermore, periodontitis has been mechanistically linked to several noncommunicable diseases, for example, cardiovascular disease, diabetes mellitus, and rheumatoid arthritis (RA). The biological mechanisms underlying and facilitating such associations are likely attributable to the indirect effects of low-grade systemic inflammation, which has been shown by us and others. The inflammatory burden can be reverted following successful periodontal therapy and has positive effects on general health.

Periodontal inflammation is evoked by the accumulation of dysbiotic microflora in connection with the gingival margin. The inflammatory response activates pathways leading to tissue destruction in individuals that are disease susceptible due to genetic polymorphisms and environmental risk factors. Gingival fibroblasts play a central role through the production of proinflammatory cytokines and proteolytic enzymes. The prolonged presence of key proinflammatory cytokines, for example, interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α), will affect the ratio between the osteoclast-stimulating factor receptor activator of nuclear factor kappa beta ligand (RANKL) and osteoprotegerin (OPG), a soluble decoy receptor of RANKL, which ultimately favors RANKL and stimulates jawbone degradation.

The established periodontitis lesion consists predominantly of lymphocytes, more precisely antibody-producing plasma B cells. The contribution of B cells to tissue destruction has been clearly demonstrated. For example, the removal of B cells in periodontitis mouse models prevents, at least partly, osteoclast numbers and leads to the formation of less osteoclastogenic cytokines. In addition, B cells express high levels of RANKL, which provides a possible mechanistic link between the infiltration and bone resorption.

Furthermore, the presence of autoreactive B cells that are activated by self-epitopes and autoantibodies targeting endogenous proteins, for example, collagen, have been demonstrated in periodontitis. The action of autoimmune B cells could explain why approximately 15% of periodontitis patients respond poorly to conventional periodontal therapy and tissue destruction progresses despite biofilm control. As targeting B cells with monoclonal antibodies in RA treatment also has positive effects on periodontal status, research focused on approaching periodontitis with host modulation therapy is motivated.

Cytokines that are important for survival and maturation of B cells are interesting candidates for future host modulation therapy. A proliferation-inducing ligand (APRIL) and B cell-activating factor (BAFF) are cytokines that interact with different B cell receptors and promote survival of plasma cells, support isotype switching, and are critical for survival and maturation of immature B cells. The overall mRNA expression of BAFF and APRIL is increased and correlates with the presence of B cells in periodontitis lesions. High serum levels of BAFF are associated with the autoimmune disease systemic lupus erythematosus (SLE), and most interestingly, concentrations of BAFF and APRIL are also increased in serum and gingival crevicular fluid from periodontitis patients. A first step to further understand the role of BAFF and APRIL in periodontitis is to explore if these cytokines are produced locally by cells in the periodontium.

In this study, we aim to explore how periodontal resident cells, especially human gingival fibroblasts, express and produce BAFF and APRIL during periodontal inflammation.

2 | MATERIALS AND METHODS

2.1 | Ethical consideration

All procedures described within this manuscript were ethically approved by the regional ethical review board at Umeå University, Umeå, Sweden, and by the Swedish Ethical Review Authority and were performed in accordance with the Declaration of Helsinki. Ethical approval for gingival biopsies and fibroblast cultures (Dnr 2013-33-31 M and 2016-417-32) and for the PerioGene North cohort (Dnr Um03-441 and 2017-487-32 M) was obtained. Verbal information was given to and written consent was received from all participants.

2.2 | Gingival biopsies

For cell culture experiments, human gingival fibroblasts were obtained from gingival papillary explants that were harvested from the keratinized gingiva under local anesthesia. The voluntary donors did not have any signs of periodontal disease, here defined as absence of bleeding on probing, probing pocket depth >4 mm, and no marginal bone loss.

For BAFF immunostaining, periodontitis tissue biopsies (n = 3) were obtained from individuals going through periodontal surgery at the specialist periodontal clinic in Västerbotten County, Sweden. Healthy periodontal tissue...
Real-time quantitative polymerase chain reaction (qPCR) was performed to analyze the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), BAFF (TNFSF13B), APRIL (TNFSF11), and interleukin-6 (IL6) with predesigned gene expression assays (GAPDH: Hs002786624_g1, BAFF: Hs00198106_m1, APRIL: Hs00601664_g1, and IL-6: Hs00174313_m1).** Samples were run in duplicates, and the data were normalized to mRNA levels of the endogenous control gene GAPDH by using the $2^{-\Delta\Delta CT}$-method. The relative alterations in gene expression of the test genes were expressed as fold increase compared to the control group at 12 h, which was set to 1. Statistical comparisons were made between the test group and control group at each timepoint and between different concentrations of recombinant protein.

2.6 | Periodontitis cohort

From the PerioGene North cohort, we selected a pilot study of 42 patients with severe periodontitis (here defined as having one tooth in each quadrant with bone loss exceeding one-third of the root length, bleeding on probing at >20% of the periodontal pockets, and having >15 remaining teeth) and 38 periodontally healthy individuals (defined as having no signs of periodontal attachment loss, probing pocket depth <4 mm at all sites, and being ≥35 years with ≥24 remaining teeth). Bleeding on probing and periodontal pocket depth was measured at six different sites per tooth. Exclusion criteria were (i) antibiotic usage or periodontal treatment in the previous 3 months, (ii) pregnancy or lactancy, or (iii) having any other known inflammatory disease or ongoing therapy with anti-inflammatory drugs. Patient characteristics are summarized in Table 1. The cohort was collected between the years 2007 and 2019, and all individuals donated blood that is stored at the Medical Biobank of Northern Sweden. A venous blood sample of 10 mL was collected from each participant into a heparinized tube at inclusion. The participants were not fasting at the time of blood sampling. Collection and handling of blood samples, including fractionation into plasma, serum, and buffy coat, and storage at –80°C followed the standardized routines at the Medical Biobank of Northern Sweden.

2.7 | Human BAFF/BLyS/TNFSF13B immunoassay

Serum from the PerioGene North cohort was used to determine protein levels of BAFF. A human BAFF/BLyS/TNFSF13B immunoassay was performed
TABLE 1 Baseline characteristics and clinical dental variables in PerioGene North.

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 38)</th>
<th>Case (n = 42)</th>
<th>p value a</th>
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<tbody>
<tr>
<td><strong>Individuals</strong></td>
<td></td>
<td></td>
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<tr>
<td>Men, n (%)</td>
<td>11 (28.9)</td>
<td>23 (54.8)</td>
<td>0.025</td>
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<tr>
<td>Women, n (%)</td>
<td>27 (71.1)</td>
<td>19 (45.2)</td>
<td></td>
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<tr>
<td><strong>Age, median [IQR]</strong></td>
<td>44.0 [37.0, 51.0]</td>
<td>54.0 [46.8–63.3]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Smoking, b n (%)</strong></td>
<td>10 (26.3)</td>
<td>30 (71.4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Number of teeth, median [IQR]</td>
<td>28.0 [28.0, 28.0]</td>
<td>25.5 [22.0, 27.0]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Bleeding on probing %, median [IQR]</td>
<td>6.0 [2.0, 14.5]</td>
<td>35.0 [25.6, 56.3]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BAFF, median [IQR]</td>
<td>483.5 [416.6–568.8]</td>
<td>532.8 [442.5–635.9]</td>
<td>0.032</td>
</tr>
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Abbreviation: IQR, interquartile range.

*a p values indicate differences between cases and controls.

*b Smoking refers to current or present smoking.

during the manufacturer’s protocol. Samples were diluted 1:2, and readings were done at 450 nm with a microplate spectrophotometer.‡‡

2.8 Immunohistochemical staining

The biopsies were placed in 4% phosphate-buffered paraformaldehyde (PFA) and subsequently embedded in paraffin. Tissue sections (5 µm thick) were deparaffinized in xylene and hydrated through a series of graded ethanol–water dilutions. Sections were rehydrated before citrate buffer heat-induced antigen retrieval steps were performed. Sections were then cooled down to room temperature and washed with PBS and PBS/Tween prior to blocking with 2.5% normal horse serum§§ for 30 min. After blocking, the tissue sections were incubated with primary antibodies—goat polyclonal anti-human BAFF/BlyS/TNFSF13B diluted to 1:12 and∥∥ goat IgG isotype control¶¶ diluted to 1:753—or as a secondary antibody control PBS was used. Sections were left to incubate overnight in a humidity chamber at 4°C. The following day, the sections were washed and treated with 0,1% H2O2 in darkness for 10 min at 4°C. Washing of the sections was performed with PBS and PBS/Tween before 1 h incubation with secondary horse anti-goat IgG antibody, ** which was visualized with 3,3′-diaminobenzidine (DAB), *** followed by nuclear counterstaining with hematoxylin. Sections were then dehydrated and mounted. Images were acquired using a light microscope††† and associated software.

2.9 Statistics

All gene expression data were tested for outliers using the Grubbs test. Since data were normally distributed, the parametric one-way analysis of variance (ANOVA) test and post hoc Bonferroni test were used. The statistical significance level was set at p < 0.05. For serum analysis, a nonparametric Mann–Whitney test was performed to compare differences between medians. BAFF levels were also analyzed in relation to periodontitis using a logistic regression model adjusted for age, sex, and smoking. Results are presented as odds ratio (OR) with 95% confidence interval (CI) and p value. The statistical significance level was set at p < 0.05.

3 RESULTS

3.1 TNF-α upregulates BAFF mRNA in human gingival fibroblasts

Human gingival fibroblasts were cultured in the presence of TNF-α (10 or 50 ng/mL) or IL-1β (30 and 100 pg/mL) for 12, 24, and 48 h. The mRNA expression of IL-6, BAFF, and APRIL was analyzed with rt-qPCR. The stimulatory effect of TNF-α and IL-1β on IL-6 expression is well established and was therefore included as a positive control. Both TNF-α and IL-1β upregulated IL-6 expression as expected (data not shown).

Addition of TNF-α at 10 ng/mL resulted in upregulation of BAFF mRNA compared to medium control at time-points 12 and 24 h (18- and 7-fold). TNF-α at 50 ng/mL upregulated BAFF at all timepoints (22-, 11-, and 9-fold). There was no statistically significant dose-dependent effect between the different concentrations of TNF-α, 10 ng/mL versus 50 ng/mL. IL-1β did not have any statistically significant effect on BAFF expression (Figure 1A).
FIGURE 1 BAFF mRNA is upregulated by TNF-α in human gingival fibroblasts. mRNA expression of (A) BAFF and (B) APRIL in human gingival fibroblasts cultured with TNF-α (10 or 50 ng/mL) or IL-1β (30 or 100 pg/mL) at 12, 24, and 48 h. mRNA expression is expressed as fold increase of endogenous control, GAPDH, at 12 h (n = 1). Graphs are representative data of three individual experiments with four replicates per group. Data are expressed as means+/standard error of the mean. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

Regarding APRIL, which was constitutively expressed at low levels, neither TNF-α nor IL-1β had any effect on mRNA expression (Figure 1B).

### 3.2 BAFF protein is enriched in periodontal resident cells in periodontitis

Since the addition of TNF-α increases the expression of BAFF mRNA in cultures of human gingival fibroblasts, we next aimed to explore the presence and distribution of BAFF protein in periodontitis tissue and in periodontally healthy control tissue.

In both periodontitis and healthy tissue, BAFF immunostaining was expressed in cells of the gingival epithelium. Positive BAFF staining in the gingival epithelium of periodontitis tissue is visualized in Figure 2A,D. What distinguished periodontitis tissue from healthy tissue was the presence of BAFF protein in the underlying connective tissue, in cells with morphologic resemblance of vessel endothelial cells and gingival fibroblasts (Figure 2B,E). Only sporadic cells positive for BAFF were detected in the underlying connective tissue in periodontally healthy biopsies (Figure 2C,F).

To rule out the possibility of the results being accountable to unspecific background staining, we performed staining on parallel sections of periodontitis tissue with BAFF antibody, isotype control antibody, and without primary antibody (see Figure S1 in online Journal of Periodontology).

### 3.3 Serum levels of BAFF are increased in periodontitis

To verify previous studies reporting on increased serum levels of BAFF in periodontitis, we analyzed BAFF protein expression in 42 periodontitis patients and 38 periodontally healthy controls from the well-characterized PerioGene North cohort. There was an uneven distribution in the proportion of men and women between cases and controls. Furthermore, the cases were older, were more frequently smokers, had fewer own teeth and a higher percentage of bleeding on probing. A statistically significant difference in BAFF serum levels was detected between periodontitis cases and controls, with a median value of 532.8 pg/mL (interquartile range [IQR] 442.5–635.9) and 483.5 pg/mL (IQR 416.6–568.8), respectively (p = 0.032) (Figure 3). However, there was no association between serum BAFF levels and periodontitis when adjusting for sex, smoking, and age (OR = 1.004, 95% CI = 0.998–1.009).

### 4 DISCUSSION

Targeting B cells, or cytokines crucial for survival and maturation of B cells, through host modulation therapy could represent a future additional treatment regime for the significant proportion of individuals with periodontitis responding poorly to standard treatment. In this study, we present novel information of how periodontal resident cells express and produce a cytokine that is vital for the function of B cells during periodontal inflammation. More specifically, we demonstrate that human gingival fibroblasts express BAFF mRNA in response to TNF-α. Further immunostaining in sections from periodontitis tissue revealed that BAFF protein was enriched in cells of the gingival epithelium and of the underlying connective tissue. Furthermore, we found that individuals with periodontitis had higher serum levels of BAFF than healthy controls in the well-characterized PerioGene North cohort.

The presence and function of BAFF and APRIL has been repeatedly documented in several chronic...
inflammatory and autoimmune diseases, such as SLE, RA, and Sjögrens syndrome. However, the role of BAFF and APRIL in periodontitis is only beginning to unravel. An earlier study by Abe and co-authors convincingly shows that the overall mRNA expression of BAFF and APRIL is increased in human periodontitis tissue homogenates. Here, we show that BAFF mRNA, but not APRIL mRNA, is increased in human gingival fibroblasts in response to TNF-α. This has, to our knowledge, not been presented before. Our findings provide an additional explanation to how the action of gingival fibroblasts can directly stimulate bone resorption as BAFF has a documented effect on osteoclastogenesis that is independent of RANKL.

Furthermore, Abe and coauthors show that APRIL protein is expressed by cells of the oral epithelium and in cells of the adjacent connective tissue. BAFF protein was found to co-localize with the presence of B cells in sections from periodontitis tissue, but not in cells of the epithelium. We detected immunostaining for BAFF within the epithelium of both diseased and healthy tissue, which is contradictory to previous results and could possibly be explained by different staining protocols and visualization methods. Increased APRIL and BAFF has also been documented in soft tissue samples of patients with peri-implantitis, an inflammatory disease characterized by destruction of peri-implant tissue. This is interesting as peri-implantitis is more frequently found in individuals with a history of periodontitis. In conclusion, BAFF and APRIL are expressed by resident and infiltrating cells in both periodontitis and peri-implantitis, and periodontal resident cells likely contribute to the overall
increased mRNA levels of BAFF observed in periodontal inflammation.

We were surprised that stimulation with IL-1β, despite using adequate doses, had no significant effect on BAFF gene expression in this study. In contrast to BAFF, APRIL mRNA was expressed at low levels in all groups and there was no upregulation neither by TNF-α nor IL-1β. Expression of BAFF and APRIL mRNA by synovial and dermal fibroblasts has been studied before. Synovial tissue samples were collected when treating patients with RA with a knee or hip replacement. The fibroblasts were stimulated by different TLR ligands and cultured for 4–72 h. Results showed increased mRNA and protein expression of both cytokines, but higher concentrations of BAFF than APRIL, which was also observed in our study. There were also different responses depending on the type of TLR stimulation, and there was a difference between synovial and dermal fibroblasts, which could explain why APRIL was not affected in our study. Another possible explanation could be that APRIL is produced by other cells in the periodontium, epithelial cells for example, or by infiltrating immune cells.

Treating RA patients with rituximab, antibodies that target B-lymphocytes, has given an observable positive effect on periodontal status. Since BAFF and APRIL are important for the function of B-lymphocytes, drugs that target these cytokines can also be considered as treatment. BAFF is important in earlier stages of development of B-lymphocytes and APRIL in later stages after antigen presentation. The effect of neutralizing both BAFF and APRIL in mouse models of experimental periodontitis has been evaluated. In one study, anti-BAFF and anti-APRIL therapy was applied in experimental periodontitis in mice. Such treatment resulted in significantly less jawbone loss, when measuring the distance between the cementoenamel junction and the alveolar bone crest. Another study investigated the effects of a BAFF-blockade in mouse experimental periodontitis with similar results. Hence, studies targeting APRIL and/or BAFF in murine experimental periodontitis present promising results.

We also show increased serum levels of BAFF in periodontitis-affected individuals compared to healthy controls, which is in accordance with previous reports. High levels of BAFF are associated with promotion of autoreactive B cells and production of autoantibodies. Targeting BAFF could have beneficial effects on regulating autoimmune responses in the treatment of several diseases. Belimumab, a BAFF inhibitor, is used to treat patients with the inflammatory disease SLE. It would be interesting to study the effects on periodontal status in these patients, which is currently unknown. Atacicept, a dual inhibitor of BAFF and APRIL, has been tested in a clinical trial in SLE patients but has not yet been approved for treatment. It is possible that dual inhibition of both APRIL and BAFF could have advantages over single inhibition. In conclusion, studies that further investigate the potential effects of inhibitors of BAFF and/or APRIL on human periodontitis and cell culture systems with human primary cells are needed.

The strengths of our study include the novelty of investigating BAFF and APRIL expression in periodontal resident cells and the fact that we used primary cells for our cell culture experiments. The limitations of this study were the fact that we were unable to determine BAFF protein in tissue and serum from the same individuals, and that we included a limited number of tissue samples without counterstaining for different cell subsets.

5 | CONCLUSION

In summary, this study shows that human periodontal resident cells participate in providing a favorable milieu for the survival and action of B cells. Periodontitis represents a complex process with interactions between local cells of the periodontium and infiltrating cells of the immune system. Targeting BAFF and APRIL and consequently inhibiting the function of B cells is a type of host modulation therapy that could be used to treat periodontitis in the future, but further research is warranted.

AUTHOR CONTRIBUTIONS

Elin Kindstedt is responsible for conception, design, and interpretation of data. Ahed Dyab, Ava Emnegard, Magnus Wänman, and Filippa Sjöström have contributed to acquisition, analysis, and interpretation of data. All authors have been involved in drafting and critically revising the manuscript. All authors have given final approval and are accountable for all aspects of the work.

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CONFLICT OF INTEREST STATEMENT
The authors declare no conflicts of interest.

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REFERENCES


**SUPPORTING INFORMATION**

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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