INCREASE OF INTRACELLULAR BAFF IN B CELLS OF SJÖGREN'S PATIENTS IS NOT AFFECTED BY DECREASE OF BAFFR

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Summary: The presence of a broad spectrum of autoantibodies in Sjögren's syndrome (SjS) patients is the result of abnormal B-cell regulation that can be at least partially explained by abnormal BAFF/BAFFR regulation. The objective of this study was to determine both membrane and intracellular expression of BAFF/BAFFR in monocytes and B-cells in peripheral blood of 19 primary Sjögren's syndrome patients and 20 healthy controls using flow cytometry. We also measured sBAFF in serum. Compared to healthy controls, both surface and intracellular expression of BAFF was significantly increased in monocytes and B-cells of SjS patients. Also serum sBAFF level was elevated. Expression of BAFFR on B-cells of SjS patients was surprisingly decreased, but there was no clear increase or decrease within monocytes. Our results indicate that activated monocytes communicate with B-cells via BAFF and BAFFR, so that B-cells are stimulated, but BAFF is also produced to stimulate cells in autocrine way. The decrease of BAFFR expression in SjS patients suggests that there is the mechanism that attempts to take over in order to balance the high level of BAFF.

Keywords: *Sjögren's syndrome; BAFF; BAFFR; intracellular expression; sBAFF*

Introduction

Sjögren's syndrome (SjS) is recognized as autoimmune exocrinopathy that primarily affects middle aged females (10, 12). Pathogenesis of SjS is caused by the immune system abnormalities. The exocrine glands are inflamed in SjS patients. Focal lymphocytic infiltrates surrounding the tubular epithelium are the hallmark of this inflammation. B-cell activity is excessive, resulting in wide spectrum of autoantibodies production (19). The pathogenesis of SjS still remains elusive with previous emphasis on the pathogenetic role of T cells. Current data indicates the substantial contribution of B-cells in the immunopathogenesis of SjS (1). B-cells may constitute germinal centers in affected salivary glands. B-cells can act as antigen presenting cells, in this way fuelling abnormal T cells response. B-cells produce not only antibodies, but also several cytokines. Numerous cytokines are indispensable for B-cell functions. Among them, the most important role is deserved to B-cell activating factor belonging to the TNFα family (BAFF). The cytokine BAFF plays a key role in B-cell differentiation, survival, and activation (16). BAFF is a ligand for three membrane receptors BCMA (B-cell maturation receptor), TACI (transmembrane activator and calcium modulator and cyclophilin ligand interactor), and BAFFR the latter receptor is being chiefly involved in BAFF signaling (11). Cells of innate immune system, including monocytes, macrophages, neutrophils, and dendritic cells, are the main producers of BAFF. Most recently, other cells that produce BAFF have been identified. These cells are of nonhematopoietic origin and include some types of epithelial cells, osteoclast, and astrocytes in CNS (6). BAFFR is widely expressed on B-cells. As for non-B-cells, BAFFR is upregulated on activated T cells and constitutively expressed on Treg subset of T cells (21). It was predicted that excessive production of BAFF shall break B-cell self-tolerance and allow self-reactive B-cells to survive. BAFF transgenic mice produce autoantibodies, leading to salivary gland destruction, the feature reminiscent of SjS (9). The serum level of soluble sBAFF is elevated in SjS patients. In addition, there is increased expression of BAFF on blood mononuclear cells (1).

Numerous biological therapies have been already approved or are in the development. These therapies aim the abnormally active soluble or membrane molecules, whose presence is a characteristic of autoimmune diseases, such as systemic lupus, rheumatoid arthritis, as well as secondary Sjögren's syndrome. Several B-cell molecules can be targeted. The most widely studied target for achieving B-cell depletion is CD20 molecule found on pre-B and mature B-cells. Two different types of BAFF antagonist are at various stages of clinical development (3). Our study is in accord

with overall effort that shows further roles of BAFF/BAFFR in the immunopathogenesis of SjS. We evaluated the expression of BAFF/BAFFR in peripheral blood monocytes and B-cells of SjS patients using flow cytometry and we also measured sBAFF in serum.

Patients and Controls

19 patients with SjS participated in this study. All of them were diagnosed with primary SjS. All patients fulfilled the European-American consensus group criteria (AECC). Diagnosis of Sjögren's syndrome was based on routine evaluation of patient's symptoms and laboratory results (autoantibodies analysis, test of salivary flow rate, Schirmer's test, medical records, etc.) performed at the Departments of Dentistry, Immunology and Allergy, Rheumatology and Ophthalmology at the University Hospital in Hradec Králové, Czech Republic. The control group enrolled in this study used no medication and consisted of 21 sex and age-matched individuals from the same geographical area. All participants confirmed their participation in the study by a written consent. The study project was approved by the Ethics Committee of the University Hospital in Hradec Králové, Czech Republic.

Tab. 1: Demographic and clinical data.

	Controls (20)	Patients (19)
Men/Women (n)	1/19	1/18
Age (years)	54	55
Symptoms of xerophthalmia (n)	0	17
Symptoms of xerostomia (n)	0	19
Dysphagia	0	14
Joint pain (n)	0	16
Thyropathy (n)	0	3
Autoantibodies (n)	0	15
NSAIDs (n)	0	4
Cystosporin A (n)	0	3
Corticosteroids (n)	0	4
Antimalarics (n)	0	1

Materials and Methods

Peripheral venous blood was collected into Vacutainer lithium heparin tubes (BD, UK). Mononuclear cells were separated from Hank's-diluted blood sample (1:1) layered over Histopaque-1077 (Sigma-Aldrich, CR). Separated cells were washed twice with PBS containing 2% FBS and 1 mM EDTA (Sigma-Aldrich, CR). Cell number was counted with hemocytometer and monoclonal antibodies were added accordingly. Monoclonal antibodies against human BAFF FITC (clone 1D6) and BAFFR PE (clone 8A7) were purchased from eBioscience, (UK) while anti-human CD14 PerCP (clone MEM-15) and anti-CD19 APC (clone LT19) antibodies were bought from Exbio, CR. Following incubation, cells were fixed with 1% paraformaldehyde for 15 min and washed. Prior to intracellular staining with antibodies (anti-BAFF, anti-BAFFR), cells were permeabilized with 0.5% saponin (5 min). Cells were washed again and measured immediately with CellQuest software on FACSCalibur (BD, USA). At least a minimum of 30,000 cells was acquired. Instrument setting and compensation was regularly adjusted using Calibrite beads with FACSComp software (BD, USA).

FlowJo software (TreeStar, USA) was used for analysis of data acquired by flow cytometry. Monocytes and B cells were distinguished on the basis of a presence of CD14 and CD19, respectively (Fig. 1). Expression of BAFF and BAFF receptor was characterized by median fluorescence intensity (MFI) that was further used for statistical analysis.

Histogram charts contain examples of flow cytometry data to show the differences between control group and group of patients. Every example that we selected was of value close to the median that described a measured parameter in a given group.

Vacutainer tubes with a thrombin additive (BD, UK) were used to collect serum samples. These samples were stored at −70 °C. sBAFF was detected using anti-human sBAFF ELISA kit purchased from R&D Systems (USA). Sensitivity of the assay was 2.86 pg/mL. Concentration of sBAFF was measured on MRX microplate reader using Revelation software (Dynatech Laboratories, USA).

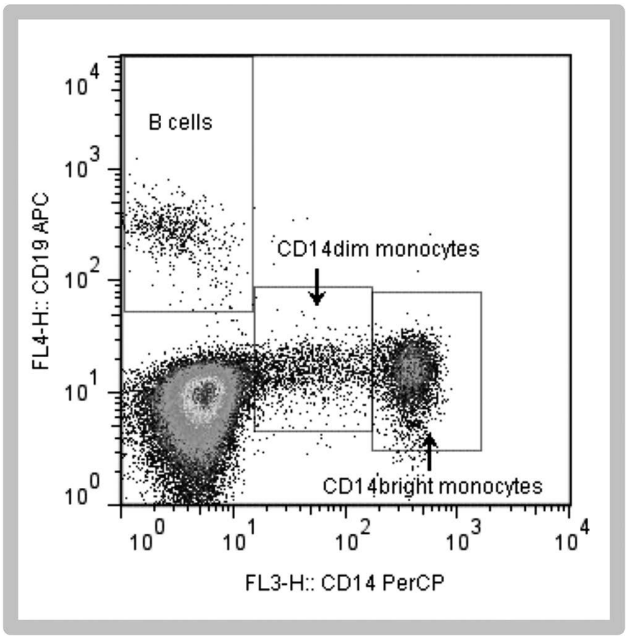

Fig. 1: Populations of cells in which expression of BAFF and BAFFR was further analyzed.

Statistical analysis

Expression of BAFF and BAFFR, and percentage of cell populations was compared between patients and controls. For this purpose, t-test or non-parametric tests (Man-Whitney test and Kolmogorov-Smirnov test) were used. Differences between clinical and demographic data were tested using χ^2 test and Fisher exact test. Before all these comparisons, normality of data sets was tested with Shapiro-Wilks test. Homoscedasticity was determined using Levene's test. All tests were performed at the 5% significance level.

Plots display median values, quartiles (box), and ranges of non-outlier values (whiskers). If not stated otherwise, any value in the manuscript represents median.

Results

Similar number of mononuclear cells was isolated from patients and controls (data not shown). When compared to control group, there was the prevalence of lymphocytes in isolated mononuclear cells of SjS patients. This difference was statistically significant (83% in control group, 87% in SjS patients, $p < 0.05$). However, the percentage

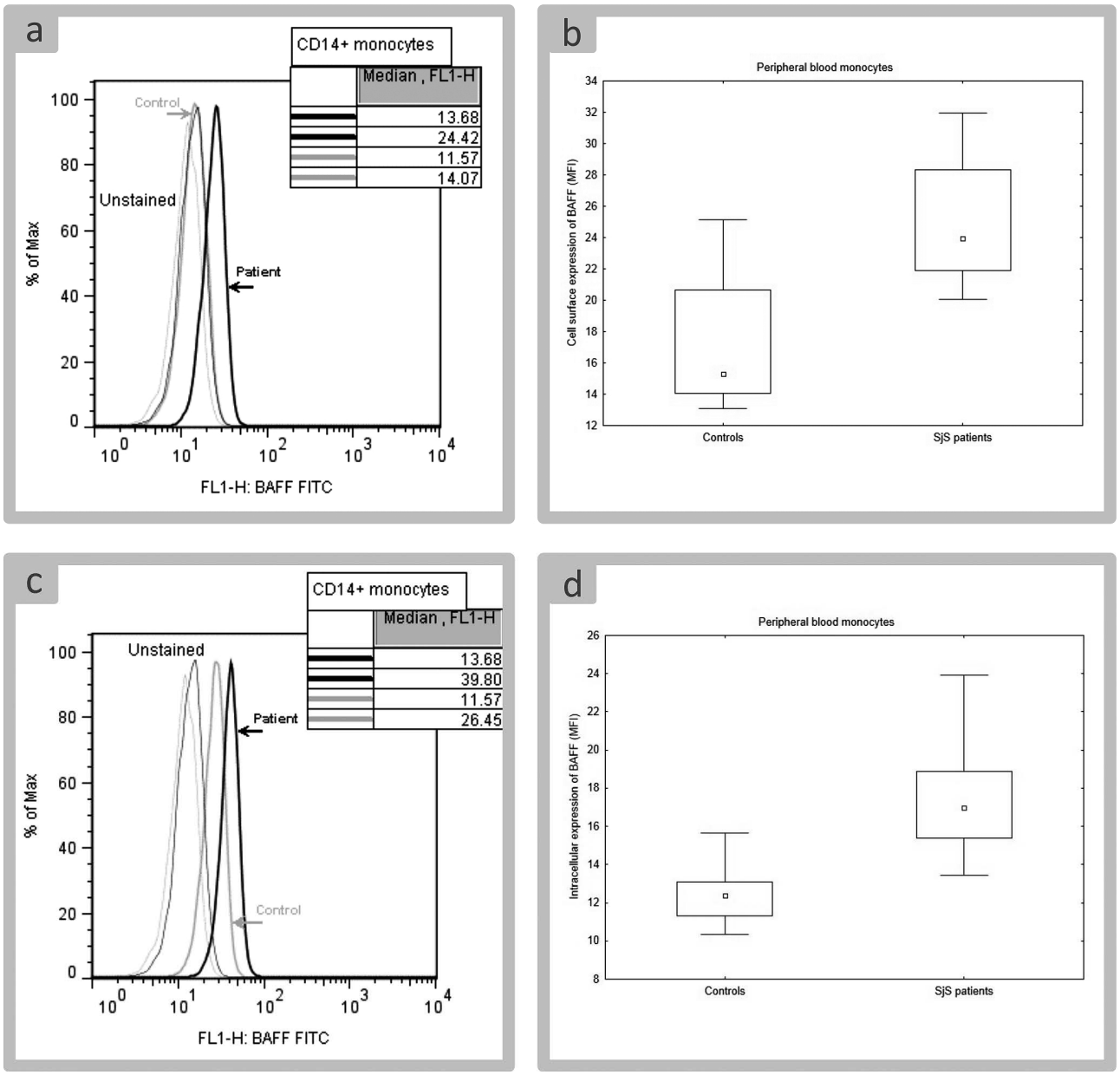

Fig. 2: Comparison between controls' and patients' BAFF expression on monocytes.

of B cells did not significantly differ between both groups (1.27% in controls and 1.71% in patients). The percentage of monocytes was higher in control group (16.2%) than in SjS patients (11.5%, $p < 0.05$). When monocytes were considered as 100% population, the percentage of monocyte subpopulations (CD14dim, CD14brigh) was not different between controls and patients.

Cell surface expression of BAFF was very low on healthy monocytes, making the increased expression in SjS patient distinctive from control group (Fig. 2A). The difference between both groups was statistically significant (Fig. 2B). BAFF was also localized intracellularly (Fig. 2C). Similarly to cell surface BAFF, intracellular BAFF was significantly increased in monocytes of SjS patients (Fig. 2C).

There was no difference in expression of BAFF between CD14dim and CD14bright monocytes, therefore, monocytes were analyzed as a unified population.

As in case of monocytes, cell surface and intracellular expression of BAFF was significantly increased in patients' B cells (Fig. 3). However, no correlation between increased BAFF on monocytes and increased BAFF on B cells was found. There was also no correlation in intracellular BAFF (data not shown).

Serum concentration of sBAFF displayed a wide range of values in patients and significantly differed from the concen-

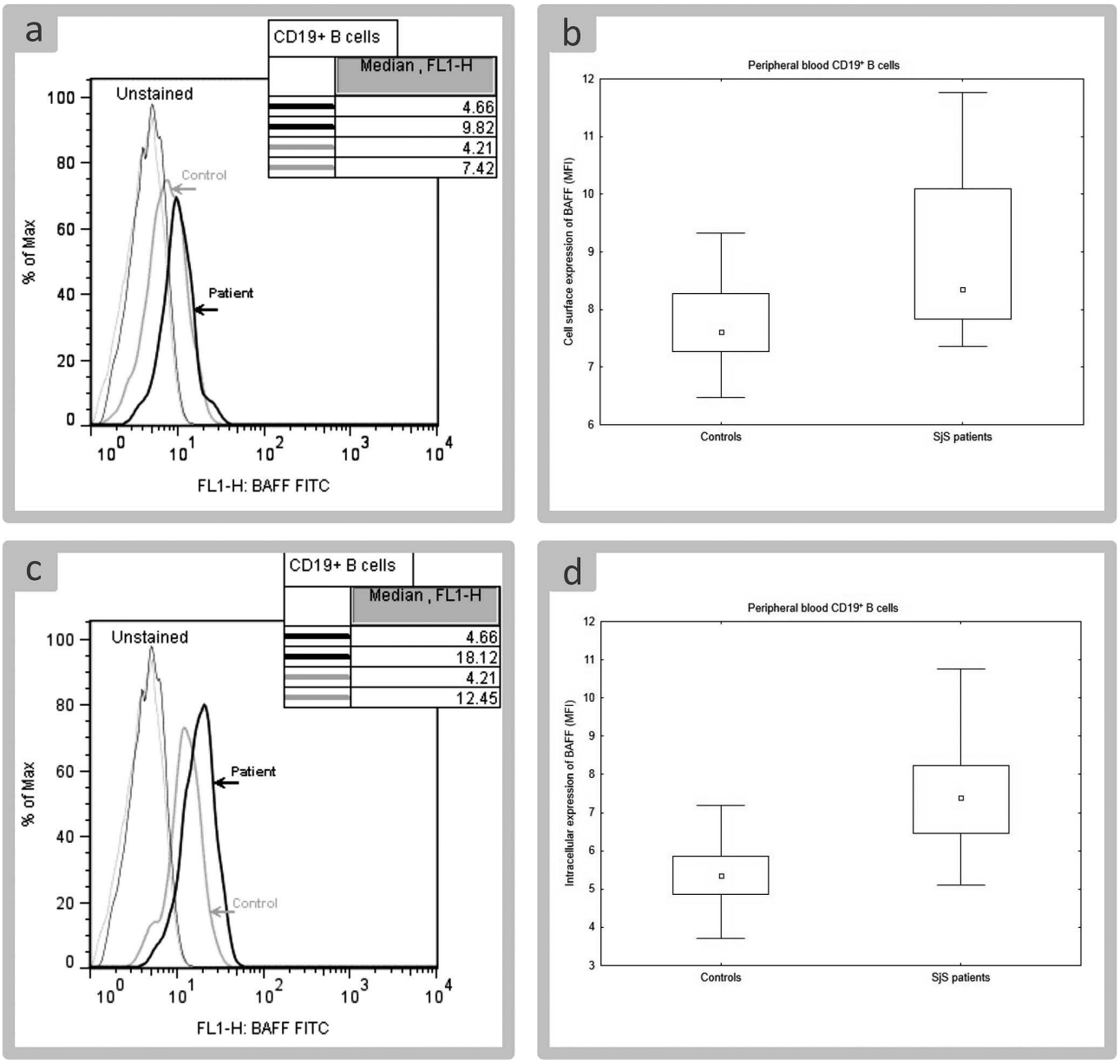

Fig. 3: Comparison between controls' and patients' BAFF expression on B cells.

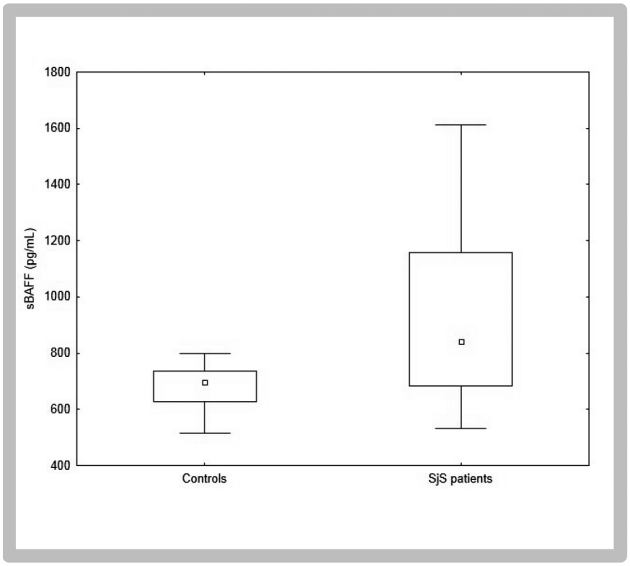

Fig. 4: Comparison of sBAFF concentration between controls and SjS patients.

tration in control group (Fig. 4). Patients who expressed high BAFF either on monocytes or B cells did not necessarily have high concentration of sBAFF; no correlation was observed between sBAFF and cell-bound BAFF (data not shown).

Expression of cell surface BAFFR on B cells was surprisingly decreased in SjS patients (Fig. 5A, B). High expression of BAFFR was also found intracellularly, but there was no difference in intracellular BAFFR between controls and patients (data not shown).

Cell surface BAFFR was expressed on monocytes, however, intracellular localization of BAFFR was not observed in this population of cells. Distribution of BAFFR values differed significantly between control group and patients (Fig. 5C). Nonetheless, due to the fact that patients' data set completely overlapped with controls, it was not possible to conclude that expression of BAFFR was increased on monocytes of SjS patients.

Discussion

The main histopathological feature of SjS is the periductal cellular infiltration of the salivary glands, predominantly by T-cells, whilst B-cells and plasma cells are commonly found in more severe lesions. Although innate immunity cells, such as monocytes, macrophages, and dendritic cells, constitute less than 5% of the total infiltrating population, they play an important role in the pathogenesis of SjS (20). Mounting evidence supports the idea that BAFF/BAFFR pathways are critically involved in abnormal B-cells activity typically seen in SjS patients. This abnormal activity demonstrates as hypergammaglobulinemia, presence of a broad spectrum of autoantibodies, ectopic germinal centre formation, oligoclonal B-cell expansion, and the well

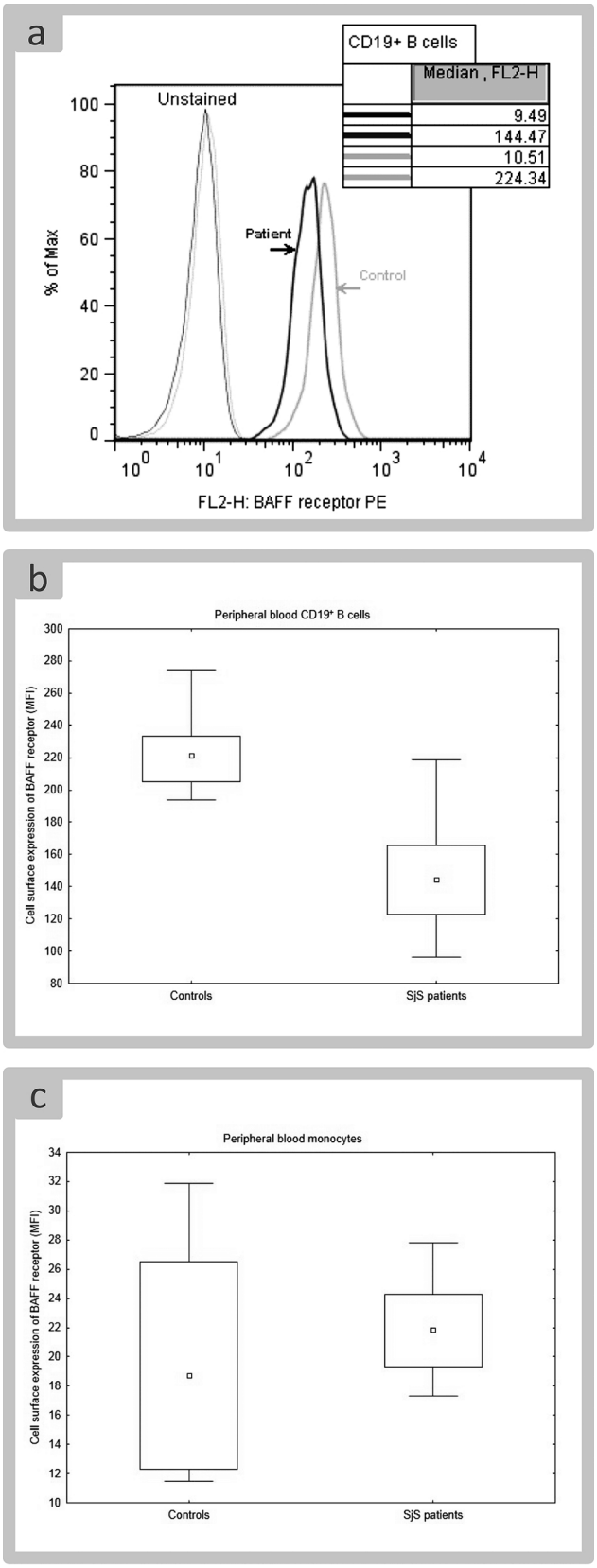

Fig. 5: Differences in cell surface expression of BAFFR.

documented risk of B-NHL lymphoma development. There are consistent reports that monocytes are contributing to the elevated of BAFF in SjS patients (8). Serum level of BAFF in SjS patients correlates with BAFF mRNA expression (2). Peripheral blood monocytes of SjS patients produce significantly higher amount of sBAFF *in vitro* both spontaneously and after stimulation with interferon γ (22). We found significantly higher presence of BAFF both on the surface and in the intracellular compartment of SjS patients' monocytes when compared to healthy controls. BAFF can contribute to B-cells physiology via its binding to the three membrane receptors of TNFα receptor family: BCMA, TACI, and BR3. BAFF interacts chiefly with BR3 which is for this reason designated as BAFFR. BAFFR is expressed on B-cells, activated T-cells, and regulatory T-cells. The expression of BAFFR is increasing as B-cells mature (21). We detected low-level expression of this receptor on monocytes of SjS patients, but no significant differences between patients and healthy control were found. In addition, no intracellular BAFFR in monocytes was identified in our study.

It is now firmly established that abnormal production of BAFF in SjS patients is linked to enhanced activity of type I interferon system (11). Increased expression of interferons I – regulated genes was described in the salivary glands, in which plasmocytoid dendritic cells were found as a principal source of interferon α. This can represent the link between innate and adaptive immunity in SjS (6). The interferons type I signature in CD14 monocytes along with higher BAFF mRNA expression identifies SjS patients with higher clinical activity of the disease (2). BAFF is a TNFα-like cytokine essential for the maturation and survival of peripheral B-cells. It exists in a membrane bound and secreted form. Cell sources of BAFF include dendritic cells, macrophages, neutrophils, activated T-cells and B-cells (4). We detected significantly enhanced membrane expression of BAFF on CD19 B-cells in blood of patients with SjS compared to healthy controls. The elevated presence of BAFF was also found in cytoplasma of SjS patients B-cells. BAFFR expression on B-cells of our patients was decreased. This is in accord with other study with no difference between naive and memory B-cells. The BAFFR down-regulation correlated with BAFF level and could be reproduced ex vivo by long-term exposure of B-cell to BAFF. The decrease of BAFFR expression on B-cells was greater in patients with extraglandular involvement than in patients with glandular involvement only (15). The decrease in the expression of BAFFR on B-cells could be explained by very likely internalization of BAFF-occupied BAFFR.

Serum level of sBAFF displayed a wide range of values in our patients and was significantly higher compared to controls. Patients who showed high expression of BAFF either on B-cells or monocytes did not necessarily have high concentration of sBAFF.

We observed no correlation between sBAFF and cell bound BAFF. Conflicting results exists for BAFF quantification. Furthermore, concentration of sBAFF fluctuates due to changes in inflammatory activity and extent of the disease, as well as the type of treatment (13, 17). In-house assays are used by some research groups. We used commercially available kit to detect sBAFF and our results are comparable with results of Quartuccio et al. (14) who reported upregulation of sBAFF in SjS patients and significant association of elevated sBAFF with development of B-cell lymphoproliferative disease.

In conclusion, BAFF controls B-cell proliferation survival, and maturation (18). BAFF is mainly produced by monocytes and excerts its function trough BAFFR. BAFFR signaling involves canonical and non-canonical NFκB pathways (5) but there is evidence that BAFFR induces other pathways (7). Our results indicate that activated monocytes communicate with B-cells via BAFF and BAFFR, so that B-cells are stimulated, but BAFF is also produced to stimulate cells in autocrine way. The decrease of BAFFR expression in SjS patients suggest that there is a mechanisms that attempts to take over in order to balance the high level of BAFF. Since the level of BAFF is still high in SjS patient's B-cells, therapy targeting BAFF is likely to bring benefits to these patients.

Dedication

This paper is dedicated to celebrate 70th anniversary of foundation of Faculty of Medicine in Hradec Králové, Charles University in Prague, Czech Republic.

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The authors declare no conflict of interest.

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