Serum BAFF levels and skin mRNA expression in patients with Behçet's disease

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ABSTRACT

Objective. Serum levels of the B-cell activating factor in the tumor necrosis factor family (BAFF), a potent contributor to B-cell survival, are elevated in patients with systemic autoimmune diseases. The objective of this study was to determine serum BAFF levels and to link the results to the clinical features in patients with skin manifestations.

Methods. Serum BAFF levels were examined by an enzyme-linked immunosorbent assay (ELISA) in 42 patients with BD (16 with active disease), 20 healthy controls, and in 20 patients with systemic lupus erythematosus (SLE) and 15 patients with multiple sclerosis (MS), who served as the disease control groups. Expression of BAFF messenger RNA (mRNA) in the skin was quantified by a real-time reverse transcription-polymerase chain reaction; the expression of BAFF receptor (BAFF-R) on CD19+ B cells was assessed by flow cytometry; and ELISA was used to evaluate the production of IgG, interleukin-6 (IL-6) and IL-10 by isolated B cells.

Results. Serum BAFF levels were elevated in patients with active BD compared to the healthy controls, and correlated positively with the extent of skin lesions. Disease remission was accompanied by decreased BAFF levels. SLE patients had the highest serum BAFF levels. Skin biopsies showed BAFF mRNA expression to be up-regulated in active BD patients. BAFF-R expression on B cells was increased in BD patients with vasculitis. Furthermore, in BD patients the ability to produce IgG and IL-6 (but not IL-10) was enhanced in BAFF-stimulated B lymphocytes.

Conclusion. These results suggest that BAFF and its signalling in B cells contribute to B cell abnormalities and the development of skin disease in patients with BD.

Introduction

Behçet's disease (BD) is a vasculitis characterized by oral and genital ulcers and uveitis. Additional target organs, with vascular, neurological and gastrointestinal manifestations, have been added to the disease spectrum (1, 2). BD may result from a vasculitis involving arterial and venous vessels of all sizes, and indeed vascular manifestations are its primary clinical feature (3). A variety of cutaneous lesions can be observed in BD and the most common (erythema nodosum-like lesions, papulopustular eruptions, superficial thrombophlebitis, and pathergy reaction) have been included in the diagnostic criteria for Behçet's disease.

The pathogenesis of BD is still unclear but immune dysfunction, viral agents and bacterial agents (such as *Staphylococcus* spp. and herpes simplex virus) have been postulated (4). Cytokines play a crucial role in the inflammatory response in BD (5-7). Previously it was suggested that there may be a possible polarization of T lymphocytes toward the Th1-type in BD (8, 9). TNF-α and IFN-γ seem to be increased in BD (10, 11) and to correlate with disease activity (12). These cytokines may act in a synergistic fashion to induce the preferential induction of Th1 cells in the BD lesion. IFN-γ is increased in mucocutaneous lesions such as oral ulcers, genital ulcers, pseudofolliculitis and lesions from the site of a positive pathergy test (13, 14).

The B-cell activating factor of the tumour necrosis family (BAFF) – also known as B-lymphocyte stimulator (BLyS); tumour necrosis family (TNF)- and ApoL-related leukocyte-expressed ligand (TALL-1); the TNF homologue that activates apoptosis, nuclear factor-κB and c-jun NH2-terminal kinase (THANK); TNF-superfamily member 13B (TNFSF13B); and zTNF4 – is a type II transmembrane molecule that is processed through furin cleavage into a biologically active soluble protein (15-19). BAFF is expressed by monocytes and macrophages, dendritic cells, and neutrophils. It binds three receptors:

BAFF-receptor (BAFF-R), which is expressed primarily by B cells (20); B-cell maturation antigen (BCMA) expressed by B cells and plasma cells (21); and transmembrane activator and calcium signal-modulating cyclophilin ligand (CAML) interactor (TACI) expressed by B and T cells (22). BAFF shares BCMA and TACI with another member of the TNF superfamily, namely a proliferation-inducing ligand (APRIL) (23). BAFF-R appears to be the most critical transducer of the BAFF-induced signals that play a central role in the BAFF system (20). Recent studies indicate that BAFF augments certain Th1 (but not Th2) responses *in vivo*, and illustrate the importance of BAFF for T-cell, as well as B-cell, responses (24, 25).

The aim of this study was to analyse whether elevated BAFF levels are a feature of BD.

Patients and methods
Patients and clinical assessment
We studied 42 patients with BD (16 patients with active disease) and 30 healthy controls. The mean age of the patients with active BD (5 females and 11 males) was 38 years (range 26-47 years) and the mean disease duration was 76 months (range 10-132 months). The mean age of the 26 patients in remission (7 females and 19 males) was 46 years (range 28-52 years). All patients met the criteria of the International Study Group for the Diagnosis of Behçet's Disease (26). Disease activity was evaluated according to published criteria (27). Two groups of patients served as disease controls: 20 patients with systemic lupus erythematosus (SLE) based on ACR criteria (28) and 15 patients with multiple sclerosis (MS) based on the diagnostic criteria of Poser *et al*. (29). The mean age of the SLE patients (4 females and 22 males) was 39 years (range 28-47 years) and the mean age of the MS patients (10 females and 5 males) was 46 years (range 33-46 years).

The clinical features of the patients with active BD are given in Table I. All 16 had active skin lesions whose extent and distribution are summarized in Table I. The skin lesions were scored using the system of Diri *et al*. (30): 0=no lesions; 1=1-5 lesions; 2=6-10 lesions; 3=11-15 lesions; 4=16-20 lesions; and 5=more than 20 lesions. Table I describes the overall disease burden in BD patients. Patients with active disease (9/16 patients) were treated with steroids and colchicines; 9 were already on treatment, while 7 newly diagnosed patients received treatment before venipuncture. Nine patients with active disease who went into remission were studied twice – once before and once after going into remission. Healthy volunteers matched for age and sex (25 men and 5 women; age range 28-47 years, mean 42 years) were included as control subjects; none of them showed any signs of acute infection or chronic disease (*e.g*., other autoimmune or atopic disorders).

Venous blood samples (20 ml) were collected from all participants aseptically into tubes with anti-coagulant. The study design was approved by our National Ethics Committee.

Table I. Characteristics of the 16 patients with active Behçet's disease.

Age/sex		Clinical manifestations	Skored skin lesion	Vascular symptoms	CNS/pulmonary involvement	RF (IU/ml)	ANA	ANCA	CRP (mg/L)
1[†]	32/M	U, OAU, GU, AE, A	[0] [†]	+	+/-	9.50	-	-	5.4
2[†]	46/M	U, OAU, GU, AE, EN, A, (+) pathergy	[1] [†]	+	+/-	9.48	-	-	7
3[†]	40/M	U, OAU, GU, AE, EN, A, (+) pathergy	[5] [†]	-	-/+	6.70	-	-	6
4[†]	26/F	U, OAU, GU, AE, EN, (+) pathergy	[5] [†]	+	+/-	7.3	-	-	5
5[†]	30/M	U, OAU, GU, AE, EN, A, (+) pathergy	[2] [†]	+	-/-	10.9	-	-	5.8
6[†]	28/M	OAU, GU, AE, A, (+)pathergy	[3] [†]	+	-/-	6.2	-	-	7
7	45/M	U, OAU, GU, AE, EN, (+) pathergy	[5]	-	+/-	8.7	-	-	9
8[†]	33/F	U, OAU, GU, AE, A, (+) pathergy	[3] [†]	+	-/-	9.5	-	-	8
9[†]	37/M	OAU, GU, AE, E, A, (+) pathergy	[3] [†]	+	-/-	7.9	-	-	3.8
10	35/M	U, OAU, GU, AE, A, (+) pathergy	[1]	-	+/-	8.2	-	-	9
11[†]	42/M	OAU, GU, AE, (+) pathergy	[4]	+	+/-	10.3	-	-	2.9
12	30/M	U, OAU, GU, AE, EN, (+) pathergy	[5] [†]	-	+/-	7.5	-	-	6
13	40/F	U, OAU, GU, AE, EN, A, (+) pathergy	[4]	+	-/+	8.7	-	-	6.5
14[†]	44/M	U, OAU, GU, AE, EN	[1] [†]	+	-/-	9.8	-	-	7.3
15[†]	41/F	U, OAU, GU, AE, (+) pathergy	[2] [†]	-	+/-	6.7	-	-	8
16[†]	47/F	U, OAU, GU, AE, EN, A	[1] [†]	-	+/-	8.6	-	-	8.2

A: arthritis; AE: acneiform eruption; EN: erythema nodosum lesions; Ul: ulcerated skin lesions; OAU: oral aphthous ulcers; GU: genital ulcers; U: uveitis; CNS: central nervous system.
[†]: Patients receiving steroid treatment. CRP: C-reactive protein (mg/L) [mean±SD: 7.2±3.6 *vs*. 1.6±1.3 in the healthy controls].
RF: rheumatoid factor (IU/ml) [8.49±1.37 *vs*. 5.36±0.87 in the healthy controls and 4.37±2.8 in the patients with BD in remission].
Antinuclear antibodies (ANA) were measured by indirect immunofluorescence on Hep2 cells with titration by serial dilution.
A titre ≥1/80 was considered positive.

Biological findings

C-reactive protein (CRP), rheumatoid factor (RF), antinuclear antibodies (ANA) and antineutrophil cytoplasmic antibodies (ANCA) were measured by indirect immunofluorescence and antigen-specific ELISA methods under routine clinical conditions.

Detection of soluble BAFF protein

Recombinant soluble human BAFF, anti-BAFF monoclonal antibody (mAb) and biotin-labeled anti-BAFF mAb were provided by Biogen Inc. (Cambridge. MA). Flat-bottom ELISA plates (Costar 9018) were coated with 50 μl anti-BAFF mAb at a concentration of 10 μg/ml in 50 mM bicarbonate buffer and stored overnight at 4°C. After removal of the coating antibody, the plates were washed thrice with washing buffer (0.2% Tween-20 in PBS, pH 7.2) and blocked with 100 μl blocking buffer (5% BSA in PBS, pH 7.2) for 30 min at room temperature. Tested samples were diluted 1:2 in dilution buffer (1% BSA in PBS, pH 7.2), 100 μl was added to each well, and the plates were incubated at room temperature for 1 hr. After 3 washes, biotin-labeled anti-BAFF mAb at a concentration of 250 ng/ml was added to each well and the plates were incubated at room temperature for 1 hr. After washing the plates 3 times, HRP-conjugated streptavidin was added at a concentration of 0.2 μg/ml, and the BAFF was incubated for 1 h. Following the addition of HRP substrate, the colour reaction was stopped using a blocking solution, and the plates were read at 450 nm. A standard curve was established using recombinant soluble human BAFF (concentration 3.04 mg/ml). BAFF values higher than +2 SD of the mean normal cut-off point were considered to be elevated.

Flow cytometric analysis

Peripheral blood mononuclear cells (PBMC) were obtained from the 16 patients with active BD. Patients receiving steroids are reported in Table I; none were being treated with immunosuppressive therapy. Two-colour analysis was performed using phycoerythrin-conjugated anti-BAFF-R (BR3 from eBioscience, San Diego, CA) and fluorescein isothiocyanate-conjugated anti-CD19 (B4 from Beckman Coulter, Miami, FL) monoclonal antibodies (mAb). For the immunofluorescence staining, samples of heparinized fresh whole blood were placed on ice immediately after collection. Blood samples (50 μl) were stained at 4°C using predetermined saturating concentrations of the test mAb for 20 minutes, as previously described by Sato *et al.* (31). Blood erythrocytes were lysed after staining using the Whole Blood Immuno-Lyse Kit, as detailed by the manufacturer (Beckman Coulter). Cells were analyzed on a FACScan flow cytometer (BD PharMingen, San Diego, CA).

RNA isolation and RT-PCR

We examined skin biopsies from the 16 patients with active BD and 10 controls (biopsy samples removed for other reasons from patients with chronic non-specific inflammation, including 2 with burn scars and 3 with decubitus ulcerations). All of the BD lesions were active at the time of biopsy (Table I). After a clinical evaluation, 4-6 mm sections of skin were snap-frozen in liquid nitrogen and stored at -80°C until used. In two patients 2 mm punch biopsies were taken from the lesions. The specimens were fixed in formalin and embedded in paraffin, sections of the paraffin blocks were stained with hematoxylin and eosin, and the biopsies were examined by a pathologist who was blinded to the patient data.

Total RNA from the skin lesions was extracted using Qiagen RNeasy spin columns (Qiagen, Crawley, West Sussex, UK) and digested with DNase I (Qiagen) to remove the chromosomal DNA, following the manufacturer's protocols. Total RNA was reverse transcribed to complementary DNA using the Promega Reverse Transcription System (Madison, WI) with random hexamers. A quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed using the TaqMan system (Applied Biosystems, Foster City, CA). BAFF probes and primers were obtained from TaqMan Gene Expression Assays (Applied Biosystems). GAPDH was used to normalize messenger RNA (mRNA), and probes and primers were taken from Pre-Developed TaqMan Assay Reagents (Applied Biosystems).

Real-time PCR was performed using an ABI Prism 7000 Sequence Detector (Applied Biosystems) following the manufacturer's instructions. The relative expression of real-time PCR products was determined using the delta delta C_t method, as previously described by Bloch *et al.* (32). A comparative threshold cycle (C_t) was used to determine the gene expression relative to a normal control (calibrator). Each set of samples was normalized with the housekeeping gene (GAPDH) using the formula $\Delta C_t = C_{t\ BAFF} - C_{t\ GAPDH}$. One of the control samples was then chosen as a calibrator, and relative mRNA levels were calculated using the term $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = \Delta C_{t\ sample} - \Delta C_{t\ calibrator}$. Hence, BAFF mRNA levels were expressed as an n-fold difference relative to the calibrator. Each reaction was performed at least in triplicate.

Production of cytokines and IgG by purified B cells

The same patients and healthy controls who underwent flow cytometric analysis were examined here. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood and B cells were isolated from the PBMC using the B Cell Isolation Kit II for humans (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, PBMC (1×10^7) was incubated for 10 minutes with biotin-conjugated mAb mixtures containing mAb to CD2, CD14, CD16, CD36, CD43, and CD235a. The B cells were then isolated by incubation with anti-biotin magnetic beads. After isolation more than 95% of the cells were found to be CD19-positive on flow cytometric analysis. Purified B cells (1×10^5) were placed in 96-well flat-bottomed plates (Becton Dickinson) and cultured in RPMI 1640 containing 10% heat-inactivated fetal calf serum (Gibco Life Technologies, Paisley, UK) at 37°C in a humidified atmosphere containing 5% CO_2. The cells were stimulated with 0.01% *Staphylococcus aureus* Cowan strain (SAC; Sigma, St. Louis, MO) plus 1 μg/ml of recombinant human BAFF (PeproTech, London,

UK), as previously described by Toubi and Shoenfeld (33).

Plates were incubated for 72 hours, and the IL-6 and IL-10 concentrations in the culture medium were measured by ELISA following the manufacturer's protocol (PharMingen, San Diego, CA). In addition, B cells were cultured for 8 days and the IgG concentrations were measured by ELISA (Bethyl Laboratories, Montgomery, TX). Each test was performed in triplicate.

Statistical analysis

Data were analysed using Sigma STAT software (SPSS, Chicago, IL, USA) and the Mann–Whitney U-tests. The level of significance was set at $p<0.05$. Correlations between serum BAFF and the extent of the skin lesions were evaluated by the rank test using Spearman's correlation coefficient. A p-value less than 0.05 was considered to be statistically significant.

Results

Elevated BAFF serum levels in active BD

Serum BAFF levels were significantly higher in the active BD patients (median: 3.4 ng/ml; range: 1.9-4.4) and the SLE patients (median: 4.4 ng/ml; range: 3.1-5) than in the BD patients in remission (median: 1.5 ng/ml; range: 0.9-3.8; p=0.0021) and the healthy controls (median: 0.65 ng/ml; range: 0.4-0.9; p=0.0038). We found no statistically significant difference in the levels of serum BAFF between MS patients (median: 0.8 ng/ml; range: 0.5-1.9) and healthy controls. BD patients in remission showed increased BAFF levels compared to the healthy control group (p=0.0262). SLE patients had higher median serum BAFF levels compared to BD patients (p=0.0001) (Fig. 1).

In active BD, BAFF levels tended to be higher in the patients with vasculitis (median: 3.98 ng/ml; range: 3.3-4.3) than in those without vasculitis (median: 2.55 ng/ml; range: 1.9-2.8; p=0.0001) (Fig. 1).

Nine patients were studied during both the active and remission stages. Four out of 5 patients receiving steroids showed decreased BAFF expression during remission, while BAFF expression
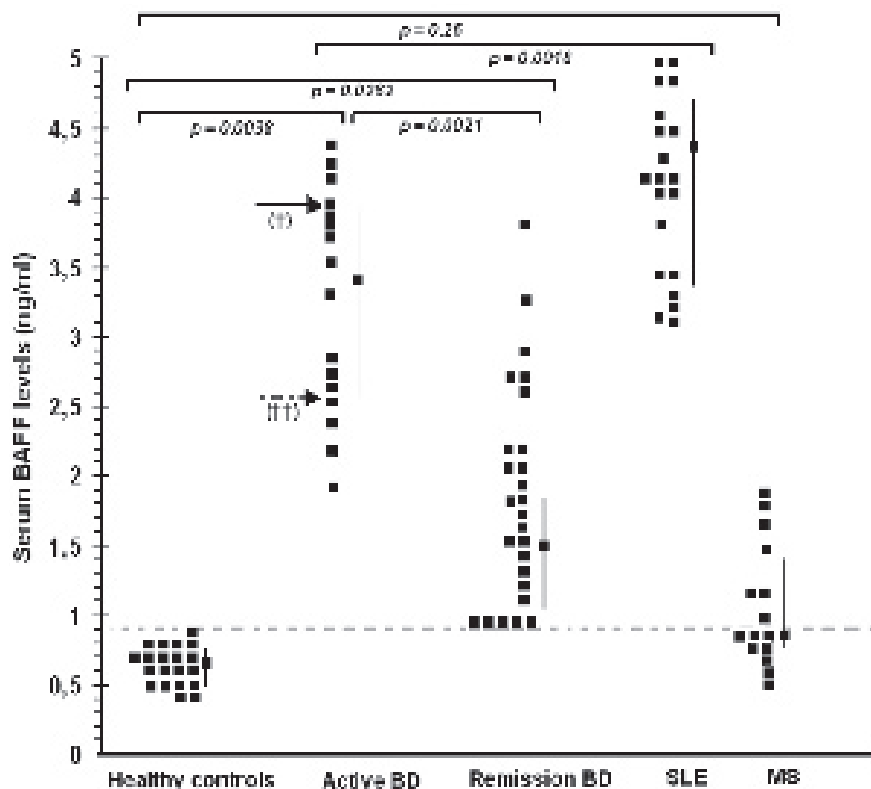

Fig. 1. Serum BAFF levels in patients with active Behçet's disease. A specific enzyme-linked immunosorbent assay was used to determine BAFF levels in serum samples from active BD patients (n=16), BD patients in remission (n=26), and healthy controls (n=20). Two groups of patients acted as disease controls; 20 with systemic lupus erythematosus (SLE) and 15 with multiple sclerosis (MS). The broken line indicates the cut-off value (mean + 2 SD of the control samples). The plotted squares indicate the values measured in individual patients. The solid arrow shows the median value for BD patients with vasculitis. The dotted arrow shows the median value for BD patients without vasculitis. Values are expressed as the median and 25th -75th percentiles. The p-values are also shown (Mann-Whitney U-test).
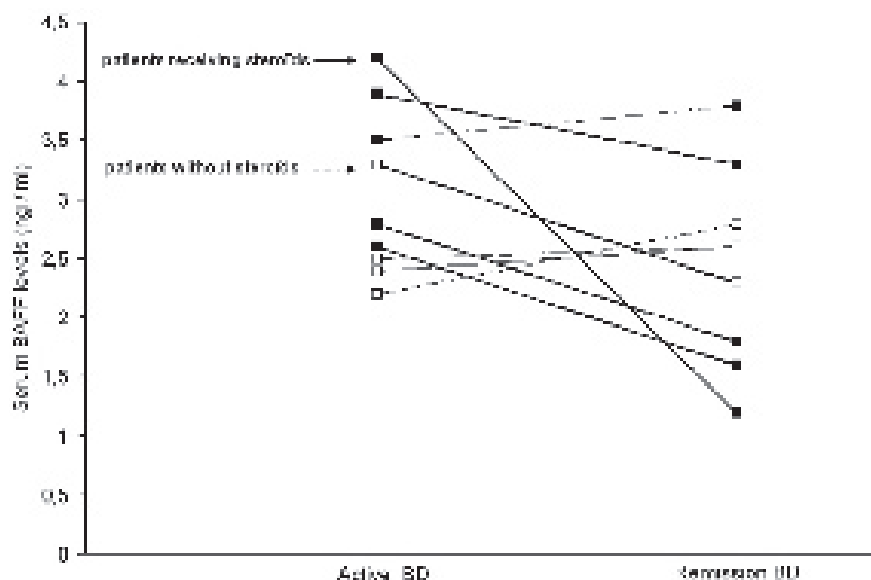

Fig. 2. Comparative analysis of BAFF levels in serum taken from 9 patients at two different time points; when their BD was active, and then after their symptoms had resolved. Five patients were receiving steroids (4/5 of whom showed decreased BAFF expression when their BD went into remission), while 4 patients were not on steroid treatment (1/4 patients showed decreased BAFF expression on remission). Statistical significance was evaluated by the Wilcoxon test.

was decreased in only one of the 4 patients not undergoing steroid treatment (Fig. 2).

CRP and RF levels were significantly ($p<0.05$; $p<0.001$) higher in active BD patients than in healthy controls. RF ($p<0.05$) and CRP ($p<0.01$) were significantly lower in BD patients in remission. There was no clear correlation between serum BAFF levels and the ANCA, CRP, RF or ANA titres in active BD (data not shown).

Clinical correlation of serum BAFF levels with skin lesions in active BD

In patients with active BD (Table I), the skin lesions were scored as 0: absent; 1-5: 2; 6-10: 3; 11-15: 4: 16-20; and 5: >20 in number (30) and the scores were correlated with serum BAFF levels. A significant positive correlation was observed in active BD between BAFF levels and skin lesions ($r=0.72$; $p=0.0001$) (Fig. 3).

BAFF mRNA expression in papulopustular lesions

BAFF mRNA expression in the papulopustular lesions of patients with active BD was significantly up-regulated compared to that in normal controls ($p=0.00016$) (Fig. 4).

BAFF-R expression on active BD B cells

BAFF-R expression on PBMC B cells from the patients with active BD was assessed by flow cytometry and the levels of expression (mean fluorescence intensity 77.6±32.9 [mean±SD]) were found to be significantly higher than those observed in healthy controls (42.6±12; p=0.00032) (Fig. 5).

Production of IL-6, IL-10 and IgG by B cells

We investigated the role of BAFF in the functioning of B cells in patients with active BD. B cells from patients and healthy controls were stimulated with BAFF plus SAC, and the culture supernatants were analyzed by ELISA to determine the amounts of IL-6, IL-10, and IgG. Unstimulated B cells from patients exhibited high levels of IL-6. B cells from patients stimulated with BAFF plus SAC produced more
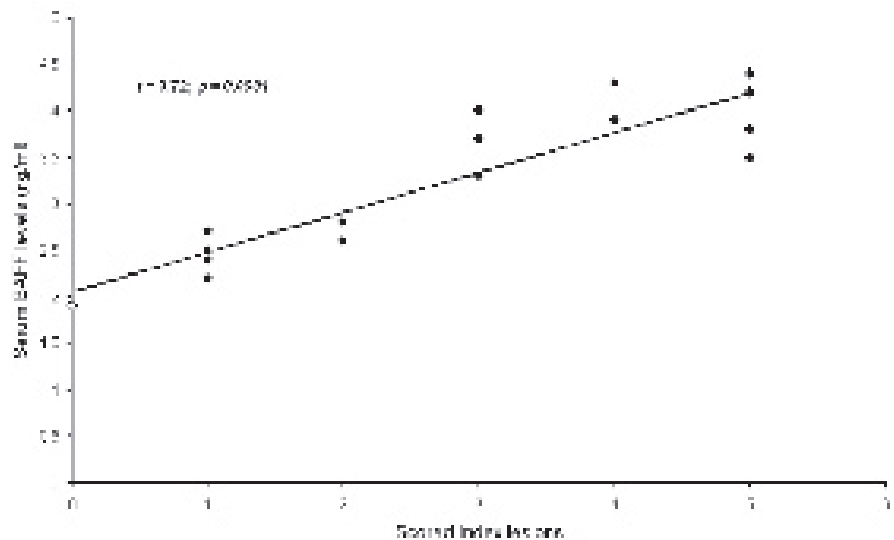

Fig. 3. Relationship between serum BAFF levels and skin lesion scores in active BD. The skin lesions were scored as: 0=absent; 1=1-5 lesions; 2=6-10 lesions; 3=11-15 lesions; 4=16-20 lesions; and 5=more than 20 lesions (20). A significant positive correlation was observed ($r=0.90$; $p<0.0001$). Histologic examination of affected skin from patients with active BD showed predominantly perivascular infiltration or leukocytoclastic vasculitis (data not shown). The correlation between serum BAFF and the extent of the skin lesions was analyzed using Spearman's correlation coefficient and the rank test. A p-value less than 0.05 was considered to be statistically significant.
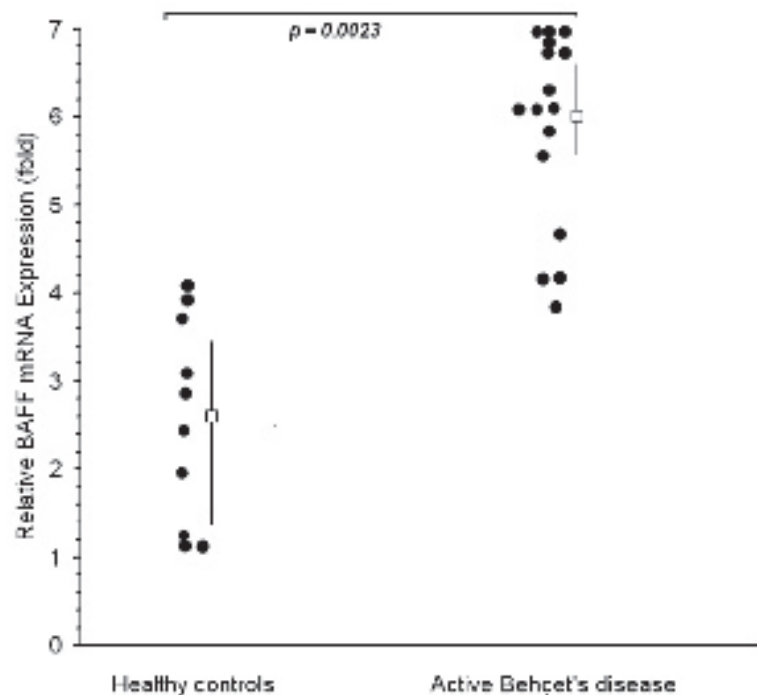

Fig. 4. BAFF mRNA expression in skin lesions from patients with active BD was measured using a real-time reverse transcription-polymerase chain reaction. Histologic examination of affected skin from these patients revealed predominantly perivascular infiltration or leukocytoclastic vasculitis (data not shown). BAFF mRNA expression relative to a calibrator (one of the control samples) was expressed as $2^{-\Delta\Delta XT}$ (fold) and is indicated by the solid circles. Data are presented as medians and 25th-75th percentiles. Statistical comparisons between pairs of groups were made using the Mann-Whitney U-test. A p-value less than 0.05 was considered to be statistically significant.

IL-6 than B cells from healthy controls, and more than the unstimulated B cells ($p=0.00024$) (Fig. 6a), whereas IL-10 production by stimulated B cells was similar between the patients and healthy controls (Fig. 6b). Furthermore, stimulated B cells from patients produced more IgG than the B cells
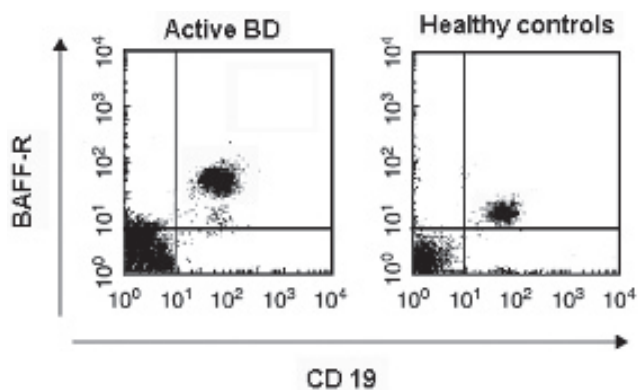
Fig. 5. Expression of the BAFF receptor on CD19-positive B cells, as assessed by 2-colour immunofluorescence flow cytometry.

Fig. 6. Production of cytokines, IL-6, IL-10 and IgG by peripheral blood B cells. PBMC samples were obtained from 16 patients with active disease (BD) and from 10 healthy controls. Purified B cells (1x10^5) from blood samples were stimulated with either medium alone or recombinant human BAFF plus *Staphylococcus aureus* Cowan strain (SAC). The culture supernatants were analyzed by an enzyme-linked immunosorbent assay to determine the levels of: (**a**) interleukin-6 (IL-6); (**b**) IL-10; and (**c**) IgG. The bars show the mean and SD. A p value less than 0.05 was considered to be statistically significant.

from healthy controls (Fig. 6c). We found no correlation between BAFF expression and IgG in the culture supernatant (r=0.127). Thus, B cells from patients with active BD had a significantly enhanced ability to produce IL-6 and IgG when they were stimulated with BAFF.

Discussion

The BAFF–BAFF-R axis appears to be the most crucial pathway for the transduction of survival signals to B cells (20) and the activation of transcription factor NF-kappa B. This study of patients with Behçet's disease is the first to demonstrate elevated serum BAFF levels and increased BAFF mRNA expression on skin biopsies. Elevated BAFF levels were associated with an increased number of skin lesions and serum BAFF levels generally decreased when the skin manifestations improved. Serum BAFF levels were found to be raised in both BD and SLE, with the latter group exhibiting the highest BAFF levels.

BAFF levels in SLE patients were similar to those reported by Matsishita *et al.* (34). In our study MS patients had similar BAFF levels to the healthy controls. In an earlier study Thangarajh *et al.* showed increased BAFF levels in MS (35), and in a recent paper they reported no augmentation in BAFF levels (36), suggesting that these findings do not play a role in MS.

BAFF levels decreased in most patients with active BD during treatment with steroids, suggesting that the steroids may have played a role. However, increased serum BAFF levels have been reported in patients with inactive BD being treated with steroids. This suggests that steroids are not the only factor in decreasing BAFF levels in BD. Similar results have been observed in systemic sclerosis (34) and SLE (37).

Cytokines play a crucial role in the inflammatory response. In particular, the significance of TNF-α and IFN-γ has been demonstrated in the development of BD (38). It is conceivable that inflammation via the production of inflammatory cytokines (39) triggers BAFF secretion in the affected tissues, which in turn stimulates B cell and, possibly, T cell responses locally.

It has been reported that B cell abnormalities could be involved in the pathogenesis of BD; increased levels of activated and memory B cell subsets suggest modified B cell functioning in BD (40). This could be related to weak stimulus by some unknown external antigen. Active BD patients were also
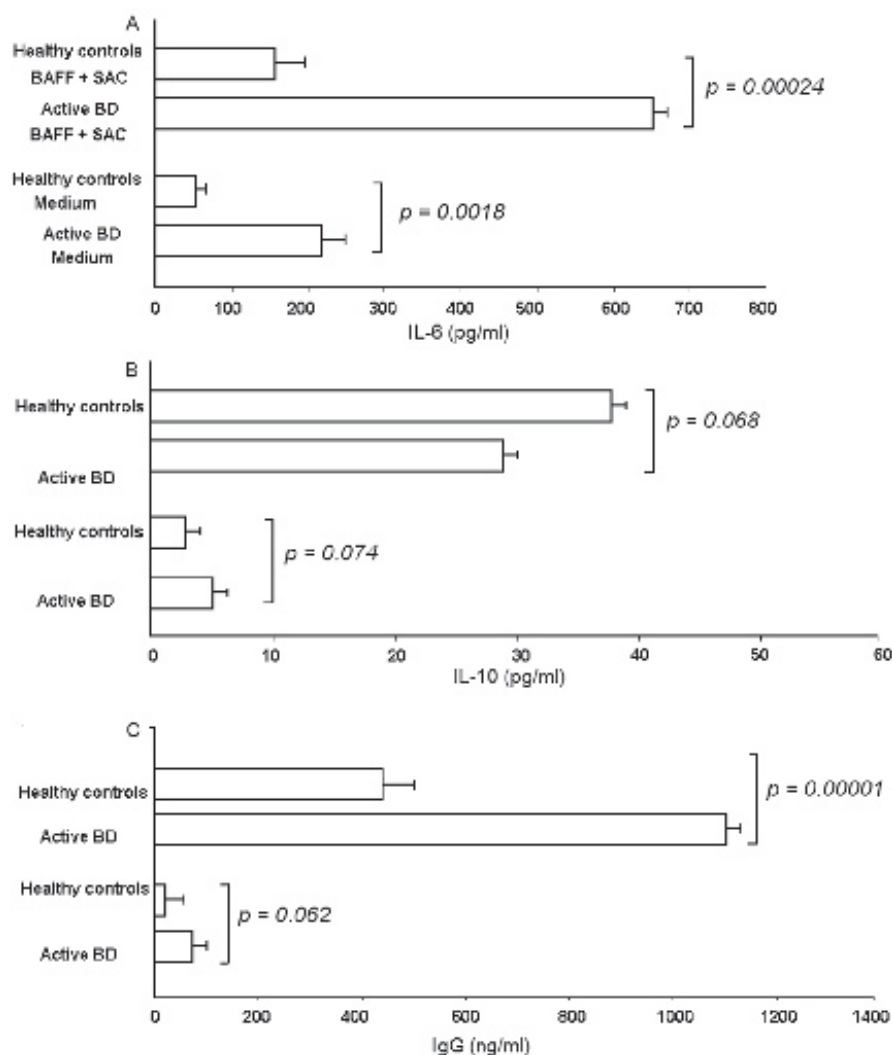
found to have elevated numbers of cells that spontaneously secrete immunoglobulin, as well as a decreased B cell response to *Staphylococcus aureus* Cowan 1 (41). BD was classified as the prototype of a viral / autoimmune / (auto)inflammatory disorder (42).

Our study shows that BAFF is up-regulated in BD, particularly in patients with skin manifestations. Baris *et al.* observed perivascular lymphocytic infiltration in the dermis of erythema nodosum-like lesions in BD patients and reported that the inflammation extended to the subcutaneous tissue in the connective tissue. They proved the role of microorganisms (*Streptococcus sanguis*, ST3, KTH-1, KTH-2, KTH-3) in the pathogenesis of BD (43). Our histological examinations yielded similar findings (data not shown).

Melikoglu *et al.* (44) compared normal skin inflammatory responses to needle-induced trauma with a hyper-reactive pathergy response in BD patients and reported that, within 48 hours after the needle prick, specific infiltrating cell populations were observed in BD. Increased influxes of mature dendritic cells, monocytes and lymphocytes (including T regulatory cells), as well as increases in cytokines (IFN-α, IL-12 p40, IL-15), chemokines (MIP3-α, IP-10, Mig, and iTac), and adhesion molecules (ICAM-1, VCAM-1), were noted after 48 hours in the skin of BD patients. These results suggest that, in contrast to the self-limiting inflammation associated with epithelial disruption in normal skin, BD patients exhibit marked cellular influxes to the injury site that lead to an exaggerated lymphoid Th1-type response. Recently it has been reported that increased levels of BAFF were associated with the new onset or worsening of organ involvement in patients with systemic sclerosis (34).

The results of earlier studies suggest that B cells are persistently activated in active BD, because the frequency of B cells expressing activation markers, including HLA-DR, is increased (40). B lymphocytes expressing CD19 constitute a critical cell surface signal transduction molecule on B cells; they regulate basal signalling thresholds and accelerate signalling via the B cell

antigen receptor (45). *In vitro* studies of the functioning of B and T lymphocytes from BD patients, using Epstein-Barr virus (EBV) as the polyclonal B cell activator, showed that IgM and IgG secretions in purified B cell cultures were increased (46). The increase in B cell activity was associated with a defective EBV-specific T cell suppressive function. Nevertheless, with a higher T:B ratio (4:1), T cells in Behçet's disease can control B cell activation (47).

In active BD patients, we found enhanced concentrations of IL-6 and IgG in BAFF-stimulated B cells. IL-6 induces concentration-dependent increases in the production of glycosaminoglycans by human dermal fibroblasts *in vitro*, and is an important mediator of inflammation (47). BAFF stimulation enhances CD19 expression and increases the ability of the B cell antigen receptor to phosphorylate CD19 (48). Thus, excess BAFF may accelerate B cell hyperactivity via the overexpression and phosphorylation of CD19 and induce B cell abnormalities in BD.

To date there are no markers to monitor Behçet's disease. Our preliminary findings suggest that serum BAFF levels could provide a useful marker of disease activity. B cells have been recently recognized as one of the therapeutic targets for systemic autoimmune diseases. In particular, BAFF has been shown to be a therapeutic target in SLE (49). Inhibition of BAFF by TACI-Ig and BAFF-R-Ig was found to be effective in treating a murine model of SLE (50). Treatment with BAFF antagonists, such as human anti-BAFF monoclonal Abs, has already been used against certain diseases and appears to be safe (51). Although the efficacy of B-cell-targeted therapy in BD is not yet known, our finding that elevated serum BAFF levels are associated with disease severity suggests that BAFF inhibition could be useful in the treatment of severe skin lesions, such as buccal and genital ulcerations.

Our data indicate that elevated BAFF levels are the result of a stimulated immune system rather than an autoimmune factor. However, the skin lesions in active BD could be caused by an interaction between T and B lymphocytes

and dendritic cells. The underlying mechanism of the inflammation in BD is not yet known. It may be genetic in nature, and BD could fall into the category of the autoinflammatory disorders – a new and expanding class of diseases characterized by recurrent episodes of systemic inflammation in the absence of pathogens, autoantibodies or antigen-specific T cells (52).

Changes in serum BAFF levels may indirectly reflect alterations in systemic inflammation with the participation of various proinflammatory cytokines, in particular IFN-α and TNF-γ, both of which are known to be potent inducers of BAFF (43). Such a scenario was demonstrated *in vivo* in a recent report by Melikoglu *et al.* (44). The levels of inflammatory cytokines that can trigger BAFF secretion in affected tissues and inflammatory sites still remain to be defined.

Our finding that elevated serum BAFF levels are associated with increased disease activity suggests that BAFF inhibition could be useful in treating BD.

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