Increased levels of BAFF in patients with Systemic Lupus Erythematosus are associated with acute phase reactants, independent of BAFF genetics: a case control study

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Running header: s-BAFF in SLE correlates with acute phase reactants

Abstract

Objectives: To determine whether increased levels of B cell activating factor (BAFF) in patients with Systemic Lupus Erythematosus (SLE) are due to disease activity or genetic variations in the promoter region of the *BAFF* gene and *BAFF* gene expression.

Methods: The case control study included 101 SLE patients and 111 healthy controls. Five single nucleotide polymorphisms (SNPs) in the *BAFF* promoter region were investigated by melting point analysis; c.-2841 (T>C), c.-2704 (T>C), c.-2701 (A>T), c.-871 (C>T) and c.-514 (A>G). BAFF mRNA levels were determined by real time PCR (BAFF-RQ) and serum BAFF (s-BAFF) levels were measured by ELISA. Independent predictors that might be correlated with increased s-BAFF in SLE patients were analysed by multivariate regression methods.

Results: Although s-BAFF levels were increased in SLE patients (1.73 vs. 0.98 ng/µl, $p<0.001$), no specific BAFF genotype was found to associate with SLE. The different genotypes defined by the investigated SNPs were identified both in SLE patients and healthy controls with similar frequencies. No association was found between BAFF genotype and BAFF-RQ. S-BAFF was independently of other factors, correlated with CRP (β 0.40, $p<0.001$), physicians visual analogue score (Rs 0.21 $p=0.046$) and inversely with haemoglobin (β -0.32, $p<0.001$) and IgA (β -0.33, $p=0.001$).

Conclusions: Increased s-BAFF levels in SLE patients are associated with the acute phase responses CRP and haemoglobin, but probably not dependent on BAFF genotype or expression. This indicates that s-BAFF production occurs at sites of inflammation.

Key words: B-lymphocyte activating factor, Systemic Lupus Erythematosus, acute phase reactants, genetics

Introduction

B-cell activating factor belonging to the tumor necrosis factor (TNF) superfamily (TNFSF13, BAFF or BLyS) is an important stimulatory factor for B-cell development and homeostasis (1). BAFF synthesis occurs in a range of immune cells (2) and can be enhanced through stimulation by inflammatory cytokines like IL-2 and INF-γ (3;4). Depending of the different receptor on mature B cells (BAFF-R, TACI and BCMA), the binding of BAFF with receptors induces Ig class switching, cell proliferation and increased survival of B cells (5). These aspects of B cell functioning are all relevant in the pathogenesis of human SLE, but most of our knowledge on BAFF originates from experimental models. Transgenic mice over expressing BAFF develop B-cell hyperplasia and hypergammaglobulinemia, a striking increase in circulating autoantibodies and immune complex mediated disease with features of SLE and primary Sjögren's syndrome (pSS) (6-8). In lupus prone mice, s-BAFF levels are increased at disease onset and blocking BAFF-dependent signals with soluble receptor prolongs their survival (9). In SLE patients, s-BAFF levels are frequently elevated and associated with disease activity including anti-dsDNA antibodies (Ab) levels (10-13). While these findings suggests that BAFF may be involved in the selective loss of B-cell tolerance in human SLE, the mechanisms responsible for increased s-BAFF levels in SLE remain unclear (5).

Genetic predisposition is an important factor in the development and expression of systemic autoimmune disease in general and characteristic B cell hyperactivity in SLE patients may be related to specific polymorphisms in B cell signalling genes (14-16). The only report on *BAFF* genetics is in Japanese SLE patients, and no associations between disease susceptibility and single nucleotide polymorphisms (SNPs) in the 5 'regulatory region of the *BAFF* gene (13q32-34) were shown (17). BAFF genotype data of non Asian SLE patients are currently lacking. However, in a study of Caucasian patients with primary Sjögren's syndrome (pSS) an association between anti-Ro/La positive patients and a specific BAFF haplotype (CTAT) was shown (18). Furthermore, the *BAFF* c.-871 T allele in the regulatory region of the *BAFF* gene was associated with increased s-BAFF in pSS

patients (18;19). Accordingly, this SNP may be involved in increased BAFF expression. In a cross sectional study on Chinese SLE patients, BAFF gene expression in peripheral blood mononuclear cells (PBMCs) was shown to be correlated with disease activity and anti-dsDNA Ab levels (20), whereas in a longitudinal North American study, BAFF mRNA was not associated with s-BAFF levels in 60% of patients (12). The discrepancy between s-BAFF levels and BAFF mRNA expression in PBMCs could indicate that in vivo BAFF production also occurs in other cells/tissue.

As there are no data that combine BAFF genotype, *BAFF* gene expression and s-BAFF with clinical data in SLE patients, we investigated if any associations exist *in vivo*, between regulatory genetic polymorphisms, BAFF gene expression and/or s-BAFF levels with regard to disease susceptibility and disease phenotype.

Materials and methods

1. Study participants

Hundred and one SLE patients (>15 years) were investigated. The patients were mainly (99%) of Caucasian descent; 87% were female, the median age was 47 years and the median disease duration was 10 years. All patients fulfilled the revised and/or updated American College of Rheumatology (ACR) criteria for the classification of SLE (21;22). Patients attended an extended clinic visit, where clinical data and biological material were taken simultaneously. Healthy volunteers (all Caucasian, 71% females and median age 48 years) were used as controls for serological investigations, gene expression studies and genotype analyses. The study protocol was approved by the local ethical committee, the national privacy agency and the Ministry of Health (ref. no 12420) and all participants gave written informed consent for the anonymous use of their data in compliance with the Helsinki Declaration.

2. Clinical categorization of patients

Lupus Nephritis (LN) designates patients with proteinuria (>0.5 g/24 hours) and/or the presence of >5 red blood cells and/or heme-granular or red blood cells casts (23). Secondary Sjögren's syndrome was defined as chronic sicca complaints with evidence of reduced tear and/or saliva production. Skin disease was defined as the presence of oral ulcers, malar or discoid rash as defined in SLEDAI. The disease activity and organ damage were scored according to SLE Disease Activity Index (SLEDAI) and Systemic Lupus International Collaborating Clinics/ACR Damage Index (SDI) (23;24). Patient and physician estimates for global disease activity were score on a visual analog scale (VAS; range 0-100mm) (25)

3. Serology

Serum BAFF levels were measured in duplex using a Quantikine Human BAFF/BLyS/TNFSF13B Immunoassay (R&D Systems, Minneapolis, USA) and results were averaged. Anti-nuclear antibodies (ANA) positive sera were routinely tested by enzyme immunoassays EIiA (VarELISA Phadia, Freiburg, Germany) for the presence of IgG subclass antibodies against double stranded DNA (anti-dsDNA), Ro (anti-SSA), La (anti-SSB), Smith (anti-Sm) anti-U1 small nuclear ribonucleoparticle (anti-U1-snRNP) and cardiolipin (aCL-G and aCL-M; normal levels <16IU/mL).

4. SNP analysis

4.1 Haplotype

We identified five common haplotypes in the 5' regulatory region within 5kb of the first exon of the *BAFF* gene (chromosome 13, 107715-107725K) with the use of Caucasian (CEU) family data from the HapMap project (www.hapmap.org) or SNP database (www.ncbi.nlm.nih.gov/snp). These haplotypes were tagged by SNP rs9514827 (c.-2841 T>C), rs3759467 (c.-2704 T>C), rs1041569 (c.-

2701 A>T), rs9514828 (c.-871 C>T) and rs3759465 (c.-514 A>G) (Figure1). Genotype and haplotype analyses were performed with SNPStats software (26).

4.2 Primers and probes

Genomic DNA was extracted from PBMCs and purified according to the instructions provided (Puregene Genomic DNA purification Kit, Gentra systems, Minneapolis, USA). Primers were designed for regions flanking each SNP. For specific detection of SNPs we used fluorescence resonance energy transfer (FRET) probes labelled with fluorescein and LC-Red 640 or LC-Red 705 (TIB Molbiol, Berlin, Germany). Primers and probes were designed using the LightCycler Probe Design Software (Roche Diagnostics, Mannheim, Germany) and the sequences are specified in the supplemental Table.

4.3 PCR and melting curve analysis

All PCR assays for SNP analysis were performed on a LightCycler1.2 (Roche Diagnostics, Mannheim, Germany) under the following conditions: 2µl DNA Master HybProbe solution (Roche Diagnostics, Mannheim, Germany), 3mM $MgCl_2$, 1µM of each primer, 0.15µM of each probe and 2µl templates in a final volume of 20µl. The SNPs c.-2841, c.-2704 and c.-2701 were analysed in a single tube using the following temperature program: Amplifications with initial denaturation at 94°C for 30sec followed by 35 cycles at 55ºC for 15sec and 72 ºC for 19sec. Directly after amplification, melting curve analysis was performed by continuous measurement of the emitted light at temperatures increasing from 35° to 80°C. The two other SNPs (c.-871 and c.-514) were analysed in separate tubes, using the same temperature program except that elongation time at 72°C was 15sec. The SNP assay findings were confirmed by sequencing of different genotypes using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA).

5. BAFF gene expression.

5.1 Design of primers and probes

Primers and probes were designed using the BAFF encoding gene *TNFSF13B* (NC_000013.10) and *B2M* gene encoding β2-microglobulin (β2M) (NC_000015.9) as templates. Primers were designed using Enhanced Avian HS RT-PCR software (Sigma-Aldrich, Saint Quentin Fallavier, France) and probes were selected using Universal ProbeLibrary Human Gene Assay (Roche Diagnostics, Mannheim, Germany) (supplemental Table).

5.2 RNA extraction and cDNA synthesis

Total RNA from frozen PBMCs was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany), following manufacturer's instructions. RNA was DNaseI treated (RNA-free), and stored at -80ºC. Total RNA (2µg) was used to synthesize cDNA in a total volume of 20µL using SuperScript VILO cDNA Synthesis Kit (Invitrogen, CA, USA) with regard of investigation full-length BAFF mRNA, according to the protocol. The quality of cDNA was then confirmed by PCR using cDNA (0.5µl), adenine phosphoribosyltransferase gene (APRT) primers (5pM) and Jumpstart ready mix Red Taq DNA polymerase (Sigma-Aldrich, Saint Quentin Fallavier, France). Thermal conditions were denaturation at 94ºC for 2min., 35 cycles at 94ºC for 10sec. 65ºC for 10sec., and 72 ºC for 15sec. and a final extension at 72ºC for 5min. Agarose gel electrophoresis were run to ensure quality of cDNA, which was stored at −20°C.

5.3 RT-PCR

Real-time PCR analysis was performed to determine the levels of BAFF mRNA in PBMCs using an ABI PRISM 7900HT, (version 2.3, Applied Biosystems, CA, USA). PCR reaction mixture contained 4µl cDNA, 10µl TaqMan MasterMix (TaqMan® Fast Universal PCR Master Mix (2x), No AmpErase® UNG, (Applied Biosystems), 5µM of each primer and probe in a final volume of 20µl.

Each analysis was performed with initial incubation at 95ºC for 20sec., followed by 40 cycles at 95ºC for 1sec. and 60ºC for 20sec. PCR reactions were done in triplicates. A range of primer concentrations was tested to ensure optimal amplification efficiency. Relative concentrations of cDNA present during the exponential phase of the reaction were determined by plotting fluorescence against cycle number on a logarithmic scale. A threshold for detection of fluorescence above background was determined. The BAFF transcript quantification was standardized using β2M as internal control. BAFF-RQ are calculated as the ratios of BAFF mRNA to β2M mRNA using the following formula: 2 exp(Ctβ2M – CtBAFF). Cut-off levels were determined by the geometric mean +2 S.D. for healthy controls (n=31). The amplification was verified by melting curve analysis and crossing point. No amplification of non-specific products was observed.

6. Statistics

As most data had a skewed distribution, numbers reported are median values unless indicated otherwise and nonparametric test methods were used in statistical analyses. Continuous data were analyzed by Mann-Whitney U test, and categorical data by Poisson distribution contingency tables or Fishers' exact test. Correlations were analyzed by Spearman rank correlation coefficients. Factors that were significant associated with s-BAFF and BAFF-RQ after univariate analyses were then entered into multiple regression models ($p<0.2$ to enter, $p>0.05$ to stay) to determine the independence of potential correlations. Statistical analyses were performed with SPSS v17.0.

Results

BAFF promoter genotype and association with SLE

In the currently studied Caucasian population the allele frequencies for the investigated SNPs in the *BAFF* gene promoter region were comparable with those reported in the HapMap database. Furthermore, the allele frequencies were similar for SLE patients and healthy controls ($p>0.2$) (Table

1). In addition, when comparing SLE patients and controls, no significant difference in genotype frequencies was found ($p>0.1$) (Table 1). As all but one individual expressed genotype AA for SNP rs3759465 (c.-514 A>G), this SNP was excluded from the haplotype analyses. The remaining four SNPs were in strong linkage disequilibrium ($p<0.00001$) and formed four common haplotypes (frequency >0.5%). No specific haplotype could be correlated with SLE and four common haplotype were found with similar frequencies in our studied SLE population and the normal controls (global haplotype association p-value=0.59) (Table 2).

BAFF promoter variation and association with BAFF gene expression

In SLE patients an increased BAFF mRNA levels was found compared to controls (RQ 1.8 ±0.63 S.D. vs. 1.1 ±0.64 S.D. $p<0.001$). However, the increased BAFF-RQ was not associated with the presence of a particular investigated SNP. In addition, increased BAFF-RQ were not associated with a specific haplotype in our investigated SLE population (p-value=0.21).

Serum-BAFF and association with BAFF promoter variation and BAFF gene expression

The measured s-BAFF levels were nearly doubled in the SLE patients (1.73ng/µl.±1.10 S.D.) compared to controls (0.98ng/µl ±0.27 S.D. $p<0.001$) (Figure 2). Single allele-, genotype- and haplotype association analyses showed however no significant association with s-BAFF values in SLE patients (data not shown). In SLE patients, no correlation was found between s-BAFF and full-length BAFF mRNA levels in PBMCs (R^2; 0.007, p=0.4) (Figure 3).

Clinical features and BAFF expression

Demographics There was no association between increased s-BAFF levels and the patients age (p=0.9) or gender (p=0.2) in the SLE cohort compared with healthy controls. SLE patients that were

daily smokers (n=34) produced significantly increased s-BAFF than non-smokers patients, (2.23 ng/µl ±1.10 S.D. vs. 1.83 ±1.10 S.D. p=0.025).

Disease activity The investigated SLE population presented with a SLEDAI 2K scores of 6 (range 0-39), while the patients VAS scores were 3 (range 0-9) and physician VAS scores were 2 (range 0-8). Physicians VAS scores correlated with s-BAFF levels (Rs 0.21, p=0.05). Single disease manifestations registered in SLEDAI and global activity scores (SLEDAI-2K, SDI) showed no significant correlation with s-BAFF or BAFF-RQ levels. The s-BAFF levels were not significantly different in patients with active renal (n=9; s-BAFF 1.86 ng/µl vs. 1.73, p=0.9), skin disease (n=21; s-BAFF 1.75 ng/µl vs. 1.73, p=0.9) or secondary Sjögren's Syndrome (n= 33; s-BAFF 1.69 ng/µl vs. 1.76, p= 0.6) compared to patients without these organ manifestation. Similarly, BAFF-RQ levels did not differ between patients with or without these organ involvements (data not shown, all p values>0.3).

Autoantibodies SLE patients with positive test of anti-dsDNA Ab (n=31) had increased levels of s-BAFF compared with patients without anti-dsDNA Ab (2.2 ng/µl vs. 1.6, p=0.009), but similar findings of increased BAFF-RQ was not found in PBMCs (1.8 vs. 1.9, p>0.4) (data not shown). Increased s-BAFF and BAFF-RQ were rather not associated with the presence of anti-Sm, anti-SSA, anti-SSB or anti-RNP Ab (data not shown). In addition, neither correlations between anti-dsDNA Ab, ELISA (OD ratio 2.0, range 0-5.8) nor anti-dsDNA Ab, ELIA (2.3 IU/mL, range 0-450) and s-BAFF levels or BAFF gene expression were found. Other autoantibodies with quantitative levels like anti-Cardiolipin Ab and Rheumatoid factor showed neither correlation, (Table 3).

Immune cells and other laboratory findings In patients with SLE, several assays in serum were measured such as CRP (4 mg/L, range 1-21), haemoglobin (Hb) (13.2 g/dL, range 8,3-17) and IgA (2.4 g/L, range 0.4-9.2). We found that s-BAFF concentrations had a strong and independent positive correlation with CRP levels (β: 0.40, p<0.001) and a negative correlation with Hb (β: -0.32, p<0.001) and IgA levels (β: -0.33, p=0.001) (Table 3). Furthermore, BAFF-RQ correlated inversely

with CD4+cells (β: -0.27, p<0.012) and IgG levels (β: -0.25, p=0.023), where median serum levels of CD4+cells were 0.55 x 10^9/L (range 0.1-1.6) and IgG 13.1 g/L, (range 5.0-27). Neither absolute complement levels of C3 and C4 nor hypocomplementemia associated with s-BAFF or BAFF-RQ levels.

Discussion

In this cross sectional study we investigated several aspects of BAFF to increase our understanding of the regulation and contribution of BAFF in human SLE. We found that genetic variation in the promoter region of the BAFF gene was not associated with SLE susceptibility, BAFF gene expression or s-BAFF. Furthermore, no linear correlation was observed between increased *BAFF* gene expression and increased s-BAFF levels. This observation together with the correlation found between s-BAFF levels and markers of the acute phase response suggest that s-BAFF production in SLE occurs mainly at local sites of inflammation.

The role of polymorphisms of the *BAFF* encoding gene has been rarely investigated in human SLE, possibly because this gene seems to be highly conserved (19;27). However, there are data suggesting that the c.-871 C>T SNP in the 5' promoter region of the *BAFF* gene increases disease susceptibility and circulating BAFF levels in patients with Sjögren's syndrome (18;19). In our study, neither this SNP nor other single or combined variations in the 5' promoter region of the *BAFF* gene, demonstrated disease association with SLE, which is in agreement with the only other study on this subject in a Japanese SLE cohort (17). The fact that *BAFF* gene polymorphisms were not reported with the main risk factors for SLE in genome wide association studies also indicates that *BAFF* polymorphisms have little impact on disease susceptibility (15;28-30).

BAFF gene expression in PBMCs was increased in SLE patients, but this was not related to the presence of distinct disease manifestations. This is in accordance with the lack of association between BAFF expression and renal disease observed in lupus prone mice that strongly express anti-

nucleosome and anti-dsDNA Ab (31). Up regulation of BAFF-mRNA has also been observed in the salivary glands of patients with primary Sjögren's syndrome. However, we were unable to confirm such association in PBMCs of SLE patients. This discrepancy may be due to difference in used biological material (blood vs. salivary cells) where mRNA was isolated from or to the fact that secondary Sjögren's syndrome developed from SLE does not lead to germinal centre formation and/or *BAFF* expression in the salivary glands (6;32). We did find a strong inverse correlation of BAFF gene expression with numbers of CD4+T-cells and IgG levels, which both are considered to be immunological signs of disease activity in SLE (33;34). This is inconsistent with Morimoto and colleagues who suggested that autoantibody production is driven by BAFF produced by T-cells and may accordingly play a pathological role in SLE (35). However, their results are based on the expression of BAFF mRNA in isolated T-cells of SLE patients which was absent in T-cells from normal controls. Since neither s-BAFF levels nor their relation with BAFF gene expression in SLE patients were reported in that study, it is not possible to determine the extent to which the contribution of BAFF production by CD4+T-cells affects the s-BAFF levels. The significant discrepancy between the BAFF gene expression and s-BAFF levels reported here have also been reported elsewhere (12) and suggest that the origin of s-BAFF is more complex. Our data underscore the hypothesis that a negative regulatory feedback mechanisms may exist between s-BAFF levels and BAFF mRNA expression in PBMCs (19). This may also be the case in a specific transgenic mouse model where it was established that BAFF exerts an anti-inflammatory effect through B cell dependent up regulation of regulatory CD4+CD25+T-cells (36).

In agreement with earlier reports, we found that almost two-thirds of studied SLE patients had increased s-BAFF levels (10;12;37). However, s-BAFF levels were neither associated with specific genotypes nor with mRNA expression levels in PBMCs and this could indicate that s-BAFF does not derive exclusively from circulating cells. The most striking findings in this study were prominent correlation between s-BAFF levels and features of the acute phase response (increased

CRP, lower albumen and Hb levels). Different inflammatory tissues have been shown to express BAFF locally (38-41), that accordingly also contributes to higher s-BAFF levels. Our data, in agreement with other studies, indicated that increased s-BAFF are closely related to systemic signs of inflammation and may not play a primary role in the autoimmune pathway of SLE (10;42).

Given the diverse nature of SLE, we stratified patients by distinct clinical features, but were unable to detect whether patients with elevated s-BAFF were more likely to have active renal- or skin disease or secondary Sjögren's syndrome. The only significant clinical correlation we found was that s-BAFF was increased in current smokers. These results support the observation that smoke inhalation may stimulate BAFF expression in epithelial airway cells (41;43;44) and emphasize the role for BAFF as a marker of inflammation. In contrast, we did not show an association between s-BAFF levels and SLEDAI scores (Rs -0.2; $p>0.8$); which is in accordance with some but not all reports (10-12;20) and may partly reflect the rather crude nature of disease activity scoring systems (45). While we could not detect which particular type of organ inflammation was associated to the increased s-BAFF levels, the close correlation between s-BAFF and anti-dsDNA Ab suggested that there is a disease specific inflammatory process involving autoantibodies triggering the increased s-BAFF levels.

There are several limitations to these results. The phenotype of SLE may vary in different populations, which implies that the findings in this Caucasian cohort can not be directly transferred to cohorts of different ethnicity. While our results do not demonstrate an influence of polymorphisms on gene expression nor on s-BAFF levels, such a role for these polymorphisms cannot be wholly excluded because of sample sizes and the possibility of a type II error. Therefore, further studies that include more patients and controls are required before a final conclusion can be made. Similar restrictions apply to our subgroup analyses, especially for patients with active renal disease. Finally, SLE is a chronic disease with intermittent disease activity and our cross sectional study design does

not allow any conclusions about variations of BAFF expression levels over time, although s-BAFF level variation seem to be limited (10;12).

In conclusion, polymorphisms in the regulatory region of the *BAFF* gene do in all probability neither contribute to the increased BAFF gene expression or increased s-BAFF levels nor to the susceptibility to SLE in Caucasian patients. There is no linear correlation between increased *BAFF* gene expression measured in PBMCs and increased s-BAFF levels. As s-BAFF levels are strongly correlated with markers of inflammation, most of the produced s-BAFF may originate from local inflamed tissues.

Key messages:

1. Serum-BAFF levels correlate strongly with acute phase reactants.
2. There is no linear correlation between BAFF gene expression in PBMCs and serum-BAFF levels.
3. BAFF gene promoter polymorphisms do not contribute to SLE disease susceptibility.

Acknowledgements We thank Kirsten Nilsen for excellent technical help and Andrea Becker-Merok, for help in ascertaining and examining the patients.

Declaration of interests The authors report no conflicts of interests.

Funding statement This work was supported by grants of Norsk Revmatikerforbund, Oslo Sanitetsforening and the Scandinavian Rheumatology Research Foundation.

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Table 1 Allele- and genotype frequency in SLE patients and controls in a Caucasian population.

SNP	Allele	SLE n=100 %	Controls n=110 %	HapMap/ SNPdatabase* %	Genotypes	SLE n=100 n (%)	Controls n=110 n (%)	p-value
Rs9514827	T	61	63	70	TT	37 (37)	43 (39)	
	C	39	37	30	CT	49 (49)	53 (48)	0.89
					CC	15 (15)	14 (13)	
Rs3759467*	T	83	85	81	TT	69 (69)	80 (73)	
	C	17	15	19	CT	30 (30)	27 (25)	0.68
					CC	2 (2)	3 (3)	
Rs1041569*	A	85	79	77	AA	76 (75)	70 (64)	
	T	15	21	23	AT	19 (19)	34 (31)	0.13
					TT	6 (6)	6 (6)	
Rs9514828	T	51	56	47	TT	24 (24)	33 (30)	
	C	49	44	53	CT	55 (55)	56 (51)	0.59
					CC	22 (22)	21 (19)	
							110	
Rs3759465	A	99	100	96	AA	100 (99)	(100)	0.97
	G	1	0	4	AG	1 (1)	0	

Table 2 Haplotype frequencies for BAFF promoter region in SLE patients and controls.

	Haplotype[a]	Total	SLE n=101	Controls n=110	OR (95% CI)	p-value
(1)	CTAT	0.37	0.38	0.36	1	
(2)	TTAC	0.28	0.29	0.27	1.02 (0.62 - 1.69)	0.93
(3)	TTTT	0.16	0.12	0.19	0.65 (0.37 - 1.14)	0.14
(4)	TCAC	0.16	0.17	0.15	1.10 (0.62 - 1.97)	0.74
	rare pool	0.03	0.04	0.02	1.37 (0.36 - 5.18)	0.64

Global haplotype association p-value: 0.59

[a]Haplotype order rs9514827 (c.-2841 T>C), rs3759467 (c.-2704 T>C), rs1041569 (c.-2701 A>T) and rs9514828 (c.-871 C>T)

Table 3 Linear regression analysis (95% CI.) of BAFF-RQ or s-BAFF and laboratory findings in SLE (n=101).

	BAFF-RQ				s-BAFF			
	Univariate		Multivariate		Univariate		Multivariate	
Feature	β	p-value	β	p-value	β	p-value	β	p-value
Anti-dsDNA ELISA	-0.16	0.57			0.10	0.66		
Anti-dsDNA ELIA	-0.30	0.18			-0.21	0.28		
aCL IgG, U/mL	0.36	0.10			0.00	0.99		
aCL IgM, U/mL	-0.45	0.08	-0.130	0.390	-0.28	0.22		
RF, IU/mL	0.24	0.19			0.21	0.20		
C3, g/L	0.19	0.33			0.19	0.26		
C4, g/L	0.08	0.69			0.01	0.98		
ESR, mm/hour	-0.31	0.25			-0.29	0.22		
CRP, mg/L	0.29	0.15			0.53	<0.001	0.398	<0.001
Hb, g/dL	-0.19	0.48			-0.37	0.10	-0.324	<0.001
Leukocyte,10^9/L	0.02	0.90			-0.26	0.10	-0.118	0.274
Tot. Lymph., 10^9/L	-0.19	0.41			0.04	0.85		
B-cells, 10^9/L	0.22	0.25			-0.08	0.63		
NK-cells, 10^9/L	-0.08	0.72			0.26	0.15	0.181	0.117
CD4+ cells, 10^9/L	-0.53	0.01	-0.271	0.012	-0.07	0.73		
CD8+ cells, 10^9/L	0.39	0.09			-0.05	0.81		
IgA, g/L	-0.23	0.22			-0.33	0.04	-0.333	0.001
IgM, g/L	0.52	0.04	0.142	0.365	0.10	0.67		
IgG, g/L	-0.45	0.03	-0.254	0.023	-0.04	0.83		
Albumen, g/L	-0.59	0.02	-0.149	0.169	-0.22	0.34		

anti-dsDNA; ELISA (OD ratio), ELIA (IU/mL), RF; Rheumatoid Factor. β; Standardized coefficients.

Supplemental Table Primers and probes used in this study. Bold capital letters; Examined SNPs

Primers and probes, name	**Sequence (5' -3')**	**Fluorescence label**
Primers and probes used for SNPs analysis		
forward primer: *rs9514827, rs3759467, rs1041569*	gaggggaacgactcac	
reverse primer: rs9514827, rs3759467, rs1041569	ttatcctggcccccaa	
anchor, c.-2841	agagaaattgctttctagcactgcgt	3' fluorescein
sensor, c.-2841	catcctttcatttc**C**gcatgtat	5' LC red 705
anchor, c.-2704,c.-2701	atggcaaggttactccgtatg	3' fluorescein
sensor, c.-2704,c.-2701	tcaccctaa**T**tg**T**tctggaac	5' LC red 640
forward primer: *rs9514828*	caacatgggagttgtagac	
reverse primer: *rs9514828*	ccttctgggactcatcac	
anchor, c.-871	aggcaaggctgattctcctca	3' fluorescein
sensor, c.-871	tagtatcatattgag**C**ggggact	5' LC red 640
forward primer: *rs3759465*	ccctccgattggattgc	
reverse primer: *rs3759465*	actgttaataaatcactctctagc	
anchor, c.-514	ttatttttatgacagcagcaggaactt	3' fluorescein
sensor, c.-514	agct**C**atctgaccttcacatt	5' LC red 705
Primers and probes used for BAFF gene expression analysis, RT-PCR		
Probe 2*, BAFF	caggagaa	5'TAMRA
BAFF forward primer	actgaaaatctttgaaccaccag	
BAFF reverse primer	ttgcaagcagtcttgagtgac	
Probe 42*, β2M	catccage	3'FAM
β2M forward primer	ttctggcctggaggctatc	
β2M reverse primer	tcaggaaatttgactttccattc	

*Universial probeLibary, human gene assays (Roche). β2M; β2-microglobulin.

Figure 1. SNPs in the 5' regulatory region of the BAFF gene used in the study.

rs9514827 (c.-2841 T>C), rs3759467 (c.-2704 T>C), rs1041569 (c.-2701 A>T), rs9514828 (c.-871 C>T) and rs3759465 (c.-514 A>G), (www.hapmap.org and www.ncbi.nlm.nih.gov/snp)

Fig. 1

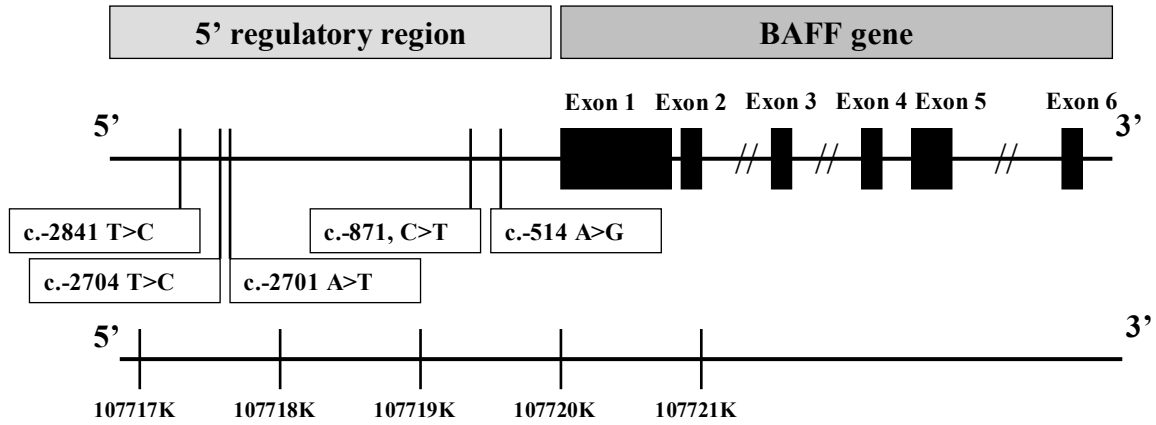

Figure 2. Serum BAFF levels in SLE patients compared with healthy controls. The results are shown in box plots. The line inside the boxes indicates the medians; the outer borders of the boxes indicate the 25th and 75th percentiles; and the bars extending from the boxes indicate the 10th and 90th percentiles.

Fig. 2

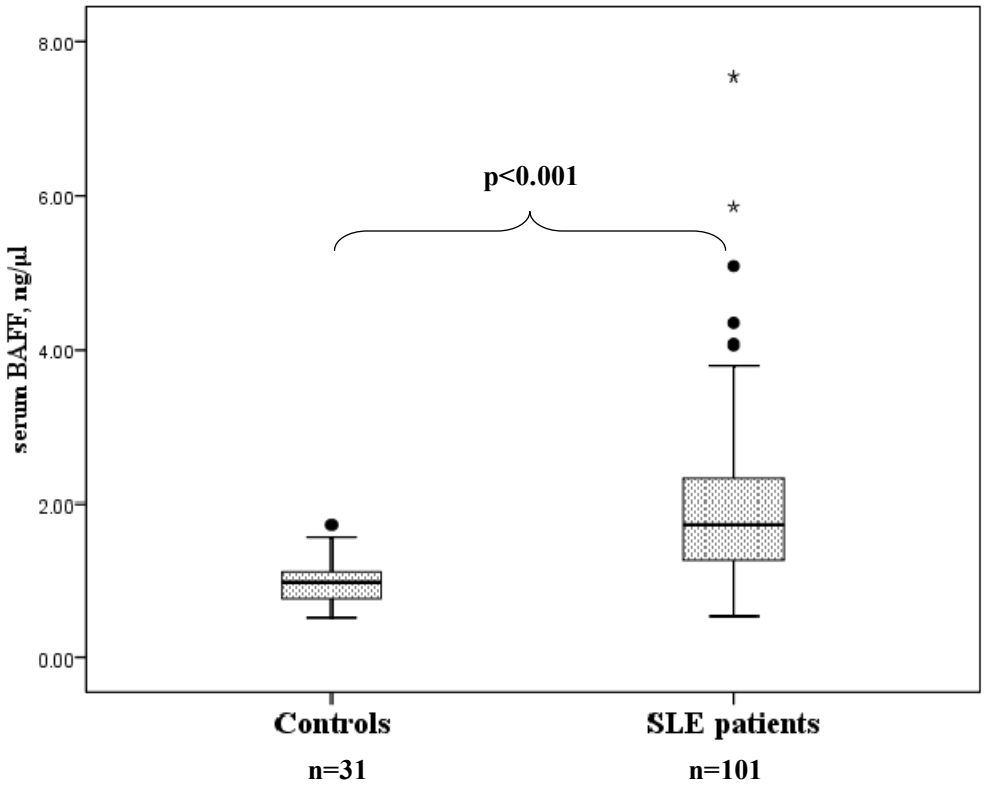

Figure 3. Low concordance between BAFF mRNA gene expression and serum BAFF in SLE patients. R^2; Correlation coefficient.

Fig. 3