BAFF and TACI Gene Expression Are Increased in Patients with Untreated Very Early Rheumatoid Arthritis

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ABSTRACT. ***Objective.*** B cells play important roles in rheumatoid arthritis (RA). Given the beneficial effect of B cell depletion therapy in RA as well as the observed alterations in B cell subpopulations in this disease, we evaluated whether changes in the expression of genes related to B cell survival and activation were already present in patients with untreated very early RA (VERA; < 6 weeks of disease duration).

Methods. The expression of a group of B cell-related activation and survival genes was quantified in peripheral blood mononuclear cells from patients with VERA by real-time PCR and compared with untreated early RA (< 1 year), established treated RA, and other untreated early arthritis conditions. Serum B cell-activating factor belonging to the tumor necrosis factor family (BAFF) was quantified by ELISA.

Results. BAFF gene expression and serum levels were highest in patients with VERA. The expression of BAFF receptor (BAFF-R) increased with disease progression, while transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) was elevated since the first weeks of RA onset. Paired box 5 gene expression was also increased at all RA stages. Chemokine (C-X-C motif) receptor 5 was elevated only in established RA. No differences were observed in B cell maturation antigen, activation-induced cytidine deaminase, B lymphocyte-induced maturation protein, and B cell lymphoma 2 expression.

Conclusion. Disturbances in the expression of B cell-related activation and survival genes, particularly BAFF and TACI, occur from the onset of RA and precede changes in BAFF-R. These alterations can lead to the development of autoreactive B cells from the first weeks of RA onset. (J Rheumatol First Release June 15 2013; doi:10.3899/jrheum.121110)

Key Indexing Terms:
RHEUMATOID ARTHRITIS B CELLS MESSENGER RNA BAFF TACI

Rheumatoid arthritis (RA) is a chronic autoimmune disease that mainly targets the joints and affects about 1% of the population worldwide[1,2]. B cells play several critical roles in RA pathogenesis. Our previous studies have demonstrated that patients with very early RA (VERA) have disturbances in peripheral blood memory B cells[3] and

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increased circulating B cell-related cytokines[4]. It has also been documented that genes regulating and affecting cellular processes such as proliferation, apoptosis, cytokine networks, and autoimmunity were differently expressed in RA B cells[5,6]. These observations led to the hypothesis that B cell biology is dysregulated from early RA development and consequently contributes to the induction and perpetuation of a pathogenic humoral immune response. Gene expression profiling of peripheral blood cells in different stages of RA progression may be useful not only to provide insights into pathogenesis, but also to identify and promote development of clinically useful biomarkers[7,8,9,10]. Therefore, the main goal of our study was to analyze the expression of a group of genes related to B cell survival, homeostasis, and activation in patients with VERA in comparison with patients in different stages of RA progression.

MATERIALS AND METHODS

Patients. Blood samples were collected from 10 consecutive patients with untreated polyarthritis (Rheumatology Department, Hospital de Santa Maria, Lisbon) of < 6 weeks' duration. After a minimum followup of 3 months, the patients fulfilled the 2010 American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) criteria for RA and were classified as having VERA. Additionally, blood samples were also obtained from 11 patients with untreated early polyarthritis of < 1 year but > 6 weeks' duration and who, after a minimum followup of 3 months, fulfilled the 2010 ACR/EULAR criteria for RA and were classified as early RA (ERA) (Rheumatology Departments, Hospital de Santa Maria, Lisbon, and Hospital da Luz, Lisbon). Blood samples were also collected from 9 patients with untreated early polyarthritis of < 1 year who did not meet the 2010 ACR/EULAR criteria for RA diagnosis and were thus classified as having other early arthritis (EA) conditions different from RA. All the patients with VERA (< 6 weeks of disease duration) were excluded if they had been treated with corticosteroids and/or disease-modifying antirheumatic drugs. The same criteria were applied to the ERA and EA groups. Further, blood samples from 21 patients with established RA treated with methotrexate (MTX) were also collected for comparison (Rheumatology Department, Hospital de Santa Maria, Lisbon). Blood samples from patients with VERA, ERA, RA, and EA were compared with 16 healthy donors used as controls. All patients and controls had blood collected in the morning in the same time period, and were fasting.

The local ethics committee approved the study and all patients gave informed consent. Patient management was in accord with standard practice and the study was conducted in accord with the Declaration of Helsinki (2008).

Measurement of autoantibodies and cytokine quantification. Rheumatoid factor (RF)-IgM was determined in all patients by ELISA kit (Human GmbH) according to the manufacturer's instructions and samples were processed using a ChemWell 2910 automated analyzer. Serum levels of anticyclic citrullinated peptide (anti-CCP) were measured by ELISA using ELIA CCP test system (Phadia GmbH) and samples were analyzed using an ImmunoCAP 100 instrument. B cell-activating factor belonging to the tumor necrosis factor family (BAFF) levels were quantified by ELISA (Bender MedSystems) according to the provider's instructions and samples were analyzed using an Infinite M200 plate reader (Tecan).

Blood cell isolation. Peripheral blood mononuclear cells (PBMC) were isolated from 20 ml heparinized whole blood following density gradient centrifugation with Percoll (Amersham). Cell counts were estimated by Trypan Blue method (Sigma-Aldrich). Cells were frozen in 1 ml/10^7 cells RPMI-1640 (Gibco), 40% fetal calf serum (Invitrogen), and 10% dimethylsulfoxide (Sigma-Aldrich) and stored at –80ºC until further use.

Separation of B cells. PBMC were isolated from 40–50 ml heparinized whole blood during a second programmed blood collection. B cells were isolated by positive MACS Separation using CD19 Microbeads and LS Columns (Miltenyi Biotec GmbH), according to the manufacturer's instructions. Purity of isolated B cells was analyzed by flow cytometry using fluorochrome-conjugated CD20 FITC (BD Biosciences) and CD3 APC (eBioscience) antibodies. A total of 10,000 cells/sample were acquired with FACSCalibur (BD Biosciences). The remaining B cells were stored at –80ºC until further use.

RNA extraction and cDNA synthesis. Total RNA from frozen PBMC and B cells was extracted using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions and treatment with RNase-free DNase Set (Qiagen) was performed to avoid contamination of genomic DNA. RNA concentration and purity were determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). RNA integrity of samples was confirmed using an Agilent 2100 Bioanalyzer with an RNA 6000 Pico Assay (Agilent Technologies) and by observing distinct 28S and 18S ribosomal bands on an agarose gel. Total RNA was reverse-transcribed into cDNA using DyNAmo cDNA Synthesis Kit for qRT-PCR (Finnzymes) with random hexamers (300 ng/µl) according to the manufacturer's instructions, and performed on a Piko Thermal Cycler (Finnzymes). The cDNA samples were stored at –20ºC.

Real-time PCR analysis. The quantitative real-time PCR (qPCR) analysis was performed on a Rotor-Gene 6000 (Corbett Life Science) using SensiMix SYBR No-ROX Kit (Bioline). The qPCR program consisted of an initial denaturation step at 95ºC for 10 min, followed by 40 cycles of 95ºC for 15 s, 60ºC for 15 s, and 72ºC for 15 s. Genes and primer sequences analyzed in our study are indicated in Table 1. Primers were designed using the Universal Probe Library Assay Design Center from Roche (www.roche-applied-science.com/sis/rtpcr/upl/index.jsp) and ProbeFinder software version 2.45. All the qPCR products were analyzed by electrophoresis in 1% agarose gels. The 18S rRNA was used as endogenous control in relative gene quantification using the comparative cycle threshold method ($2^{-\Delta\Delta Ct}$)[11]. All data were analyzed with Rotor-Gene 6000 Series Software version 1.7.87.

Statistical analysis. Statistical differences were determined with GraphPad Prism. For populations that did not follow a Gaussian distribution, nonparametric tests were used. The Mann-Whitney U test was used for comparisons between 2 independent groups. For comparisons between 3 or more groups, the Kruskal-Wallis and Dunn's multiple comparison tests were used. Correlation analysis was performed using Spearman's test. Differences were considered statistically significant for $p < 0.05$. To counteract the problem of multiple comparisons with 10 gene analysis, the Bonferroni correction was applied and p value < 0.005 set as statistically significant.

RESULTS

Characterization of patients and disease evaluation. Patients at 3 different clinical stages of RA were included in our study. Patients with untreated VERA (n = 10) of < 6 weeks' duration had a mean ± SD age of 54 ± 18 years, 80% were women, 60% were RF-positive, 50% were anti-CCP-positive, and 40% of patients were seronegative for both RF and anti-CCP. The baseline 28-joint Disease Activity Score (DAS28) was 7.1 ± 0.9. These patients represent a subset of a larger cohort previously described by our group[3]. The patients with untreated ERA (n = 11) had a mean disease duration of 0.7 ± 0.4 years and a mean age of 53 ± 5 years. Eighty-two percent were women, 55% were

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Table 1. Genes and sequences of primers used in quantitative real-time PCR.

Gene	Name	Primer Sequences
BAFF	B cell activating factor	Fw: 5'-GAG AAG CTG CCA GCA GGA-3' Rv: 5'-GGA GCT GGT GGT TCA AAG ATT-3'
BAFF-R	BAFF receptor	Fw: 5'-CTG GTC CTG GTG GGT CTG-3' Rv: 5'-ACC TTG TCC AGG GGC TCT-3'
TACI	Transmembrane activator and calcium modulator cyclophilin ligand interactor	Fw: 5'-AGG CTC AGA AGC AAG TCC AG-3' Rv: 5'-CCA GGA AGC AGC AGA GGA-3'
BCMA	B cell maturation antigen	Fw: 5'-AGG ACG AGT TTA AAA ACA CAG GA-3' Rv: 5'-TCA CAG GTG CAT TCT TCC AC-3'
AID	Activation-induced cytidine deaminase	Fw: 5'-GGA CTT TGG TTA TCT TCG CAA T-3' Rv: 5'-GTC GGG CAC AGT CGT AGC-3'
CXCR5	Chemokine (C-X-C motif) receptor 5	Fw: 5'-GCC ATG AAC TAC CCG CTA AC-3' Rv: 5'-TCT GTC CAG TTC CCA GAA CA-3'
Blimp-1	B lymphocyte-induced maturation protein	Fw: 5'-ACG TGT GGG TAC GAC CTT G-3' Rv: 5'-CTG CCA ATC CCT GAA ACC T-3'
Pax5	Paired box 5	Fw: 5'-GCC AAA ATC CCA CCA TGT-3' Rv: 5'-GTG GCT GCT GTA CTT TTG TCC-3'
$ß_2m$	$ß_2$-microglobulin	Fw: 5'-CTA TCC AGC GTA CGC CAA AGA TTC-3' Rv: 5'-CTT GCT GAA AGA CAA GTC TGA ATG-3'
Bcl-2	B cell lymphoma 2	Fw: 5'-TTG ACA GAG GAT CAT GCT GTA CTT-3' Rv: 5'-ATC TTT ATT TCA TGA GGC ACG TT-3'
18S rRNA	18S ribosomal RNA	Fw: 5'-GGA GTA TGG TTG CAA AGC TGA-3' Rv: 5'-ATC TGT CAA TCC TGT CCG TGT-3'

RF-positive, 45% were anti-CCP positive, 36% were seronegative for both RF and anti-CCP, and their mean DAS28 score was 5.4 ± 1.2. Patients with established RA (n = 21) had an average disease duration of 11.5 ± 10.6 years, were mostly under MTX monotherapy (n = 17), or were receiving combination therapy with MTX and sulfasalazine (SSZ; n = 2), MTX and hydroxychloroquine (HCQ; n = 1), or MTX, SSZ and HCQ (n = 1). The mean age of these patients was 57 ± 14 years. The group was 76% women, 71% were RF-positive, 65% were anti-CCP-positive, 24% were seronegative for both RF and anti-CCP, and the mean DAS28 score was 4.5 ± 1.6 (Table 2). The EA group included 9 patients, with several different diagnoses: systemic lupus erythematosus (3 cases), spondyloarthritis (2), arthritis associated to dermatomyositis (1), arthritis associated to human immunodeficiency virus infection (1), and unremitting undifferentiated arthritis (1). One patient who entered spontaneously into remission before 3 months of followup remained without a specific diagnosis and was thus classified as presenting a self-limited form of polyarthritis. Patients with EA had a mean disease duration of 0.2 ± 0.3 years and a mean age of 40 ± 16 years, 89% were women, all were RF-negative and anti-CCP-negative, and the initial DAS28 was 5.4 ± 0.8 (Table 2). The details of

Table 2. Clinical information for patients with EA, VERA, ERA, and RA. Data are mean ± SD unless otherwise stated.

Characteristic	Controls, n = 16	EA, n = 9	VERA, n = 10	ERA, n = 11	RA, n = 21
Age, yrs	50 ± 11	40 ± 16	54 ± 18	53 ± 5	57 ± 14
Sex (% female)	69	89	80	82	76
Disease duration, yrs	NA	0.2 ± 0.3	< 6 weeks	0.7 ± 0.4	11.5 ± 10.6
CRP, mg/dl	ND	2.5 ± 2.5	2.3 ± 2.3	2.1 ± 1.8	1.4 ± 1.6
ESR, mm/h	ND	44.1 ± 28.8	57.2 ± 36.7	47.7 ± 40.0	24.2 ± 13.8
DAS28	NA	5.4 ± 0.8*	7.1 ± 0.9	5.4 ± 1.2	4.5 ± 1.6*
RF-positive (%)	ND	0	60	55	71
Anti-CCP-positive (%)	ND	0	50	45	65

* Differences were considered statistically significant for p values < 0.05 in comparison with patients with VERA. VERA: very early rheumatoid arthritis; ERA: early rheumatoid arthritis; RA: rheumatoid arthritis; EA: early arthritis; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; DAS28: 28-joint Disease Activity Score; RF: rheumatoid factor; anti-CCP: anticyclic citrullinated peptide; NA: not applicable; ND: not determined.

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clinical measures for individual patients in all groups are available from the author on request.

Patients with RA have gene expression changes in peripheral blood mononuclear cells from the first weeks of disease development. A group of genes related to B cell homeostasis and survival [BAFF, BAFF receptor (BAFF-R), transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI), B cell maturation antigen (BCMA)], class-switching [activation-induced cytidine deaminase (AID)], chemotaxis [chemokine (C-X-C motif) receptor 5 (CXCR5)], plasma cell differentiation [paired box 5 (Pax5) and B lymphocyte-induced maturation protein (Blimp-1)], immune system activation [β_2-microglobulin (β_2m)], and apoptosis [B cell lymphoma 2 (Bcl-2)] was analyzed in 3 independent groups of patients with RA representing different stages of disease progression. The disease stages were classified as < 6 weeks' mean disease duration (VERA), < 1 year (ERA), and 12 years (established RA). Results were compared with EA patients and healthy controls. Disturbances in gene expression levels of BAFF receptors were found in both early and established RA in comparison with controls. BAFF-R gene expression was elevated in ERA and RA in comparison with controls, while no significant differences were observed in VERA and EA (Figure 1A). Importantly, patients with established RA had higher BAFF-R gene expression levels when compared to VERA, which suggests that BAFF-R expression increases with disease progression. Further, TACI gene expression levels were significantly increased in patients with VERA, ERA, and RA in comparison with controls, while no differences were detected between EA and controls (Figure 1B). BCMA gene expression levels were similar in all patients compared to controls and no significant differences were detected (data not shown). BAFF gene expression was highest in patients with VERA in comparison with controls, RA, and EA (Figure 1C). This early increase in BAFF mRNA expression observed in VERA was not observed in patients with EA. In contrast, CXCR5 gene expression was higher in patients with RA not only in comparison with controls, but also in patients with VERA (Figure 1D). Additionally, Pax5 gene mRNA levels were elevated in all patients with RA in comparison with controls (Figure 1E). Of note, patients with EA had a significantly lower expression of Pax5 in comparison with established RA. The gene expression levels of β_2m were significantly elevated in patients with VERA, ERA, and EA in comparison with controls and established RA (Figure 1F). Moreover, no significant differences were observed in AID, Blimp-1, and Bcl-2 gene expression (data not shown).

Because evaluation of the expression of a group of B cell-related genes has been performed in PBMC, including not only B cells but also T cells and monocytes, the differences in gene expression could reflect the different proportions of cells present in the peripheral blood of controls and patients with RA in different disease stages, although the number of total PBMC frozen per sample was similar in all groups. Of note, the frequency and absolute leukocyte counts in blood, which included lymphocytes (B and T cells), neutrophils, and monocytes, were not significantly different in patients with RA when compared to controls[3]. Similarly, in our study, the frequencies of all circulating cell populations were not significantly different between patients with RA in diverse disease stages and healthy controls (data not shown).

B cells have increased TACI and CXCR5 gene expression at later stages of RA development. To validate our observations specifically on isolated B cells, a second blood collection for B cell separation and gene expression analysis was done for patients with RA and healthy controls. Blood was collected from 8 individuals from the initial group of 21 patients with established RA, because 6 patients could not be evaluated owing to commencement of biological therapy (3 with tocilizumab and 1 with rituximab), 6 were not available for an additional blood collection, and 1 had a chronic skin infection. All the patients with established RA who agreed to participate in the second blood collection had average disease duration of 10.4 ± 6.9 years and were receiving MTX monotherapy close to the time of the first blood collection, except for 1 patient who was simultaneously receiving MTX and SSZ. Further, 63% were women, 75% were RF-positive, and 75% were anti-CCP-positive. The mean C-reactive protein (CRP) value was 2.1 ± 1.0 mg/dl, the erythrocyte sedimentation rate (ESR) was 40.5 ± 22.2 mm/h, and the mean DAS28 score was 4.4 ± 1.3. Blood was collected from patients with established RA for the second time after a mean of 11 months from the first blood collection. A second blood collection was obtained from 10 of the initial 16 healthy controls because of the unavailability of the remaining 6 controls. Seventy percent of the controls who repeated the blood collection were women.

Similarly to what has been observed for PBMC, isolated B cells from patients with established RA had increased gene expression levels of TACI and CXCR5 (supplementary figure available from the author on request). In addition, BAFF-R and Pax-5 gene expression showed a tendency toward higher values in patients with established RA, although this result was not statistically significant. Further, no significant differences could be detected in β_2m and BAFF gene expression (supplementary figure available from the author on request), or in the mRNA levels of AID, BCMA, Blimp-1, and Bcl-2 (data not shown) as previously observed for PBMC.

Correlation analysis between gene expression, serum BAFF levels, and clinical data. Because there were alterations in relative gene expression of BAFF-R not only in patients with early RA, but also in established RA, BAFF serum levels were quantified in all groups of patients to analyze a

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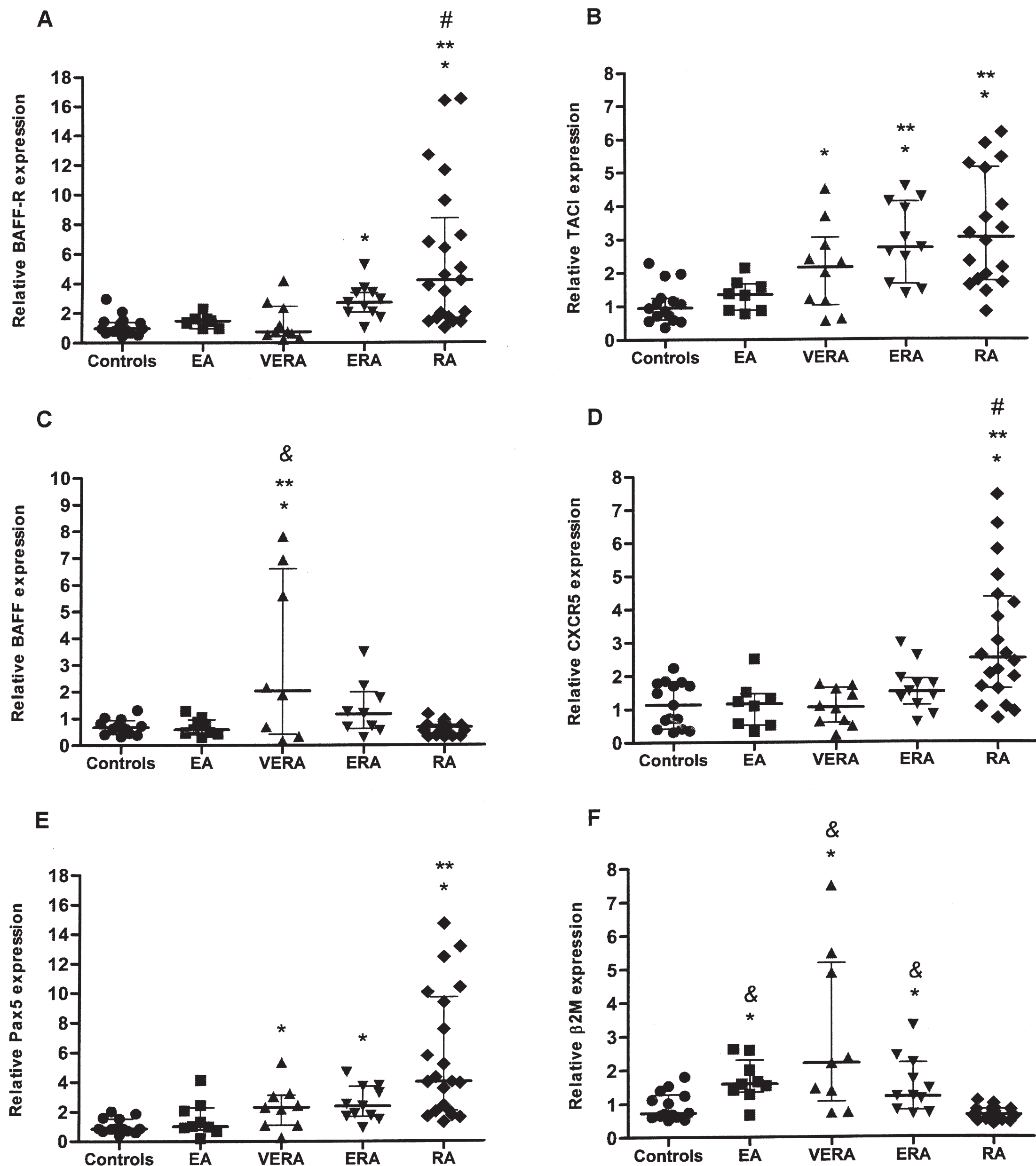

Figure 1. Patients with very early rheumatoid arthritis (VERA) have increased gene expression levels of B cell-activating factor belonging to TNF family (BAFF), transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI), and β_2m in comparison with controls. Relative gene expression from peripheral blood mononuclear cells was determined by real-time PCR in patients and controls. The relative mRNA quantities ($2^{-\Delta\Delta Ct}$) of (A) BAFF-R, (B) TACI, (C) BAFF, (D) CXCR5, (E) Pax5, and (F) β_2m are indicated. A nonparametric statistical analysis (Kruskal-Wallis and Dunn's multiple comparison tests) was performed: bars represent median relative gene expression values with interquartile ranges. Differences were considered statistically significant for $p < 0.005$ with Bonferroni correction for all genes analyzed (BAFF-R, TACI, Pax5, and β_2m: $p < 0.0001$; CXCR5: $p = 0.0006$), except for BAFF ($p = 0.0066$) in comparison with *controls, **EA, #VERA, and &RA.

possible correlation between the cytokine production and its receptors' mRNA expression. Serum samples from 30 healthy controls (mean age 51 ± 10 years, 67% women) were used for comparison. Patients with VERA had the highest BAFF serum levels in comparison with controls, ERA, and RA patients (Figure 2), but no correlation could be observed either with BAFF-R, TACI, or BCMA gene exprssion (data not shown). Also, patients with EA had increased BAFF serum levels in comparison with ERA. Further, no correlation was found between BAFF serum levels and age, DAS28, ESR, CRP, swollen and tender joint counts, or any of the genes analyzed for all patients (data not

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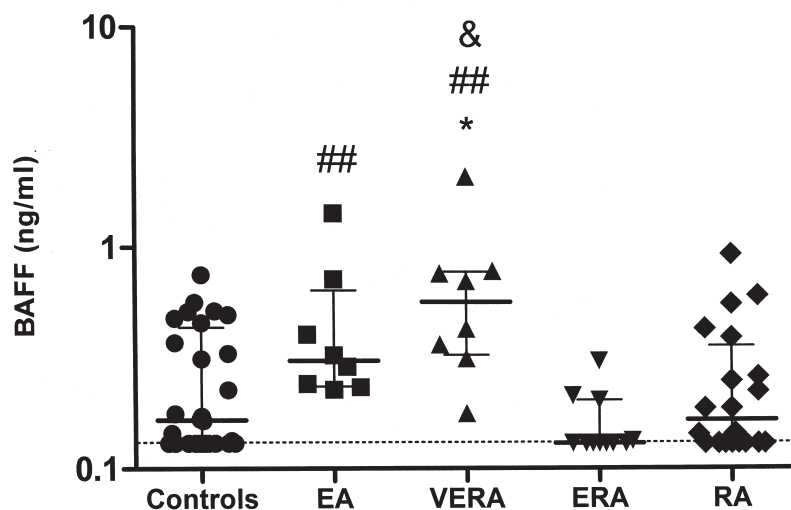

Figure 2. Levels of serum B cell-activating factor belonging to TNF family (BAFF) are elevated in very early rheumatoid arthritis (VERA) compared to other patients with arthritis and to controls. Serum BAFF levels were determined by ELISA in all patient and control samples. Nonparametric statistical analysis (Kruskal-Wallis and Dunn's multiple comparison tests) was performed: bars represent median values with interquartile ranges (p = 0.0005). Broken line represents limit of detection for the assay (0.13 ng/ml). Differences were considered statistically significant for $p < 0.005$ with Bonferroni correction in comparison with *controls, ##ERA, and &RA.

shown). Moreover, to verify whether an association existed between B cell-related gene expression results or serum BAFF levels and the presence of autoantibodies in serum, the relative gene expression of all genes included in the study and circulating BAFF levels were analyzed in all patient groups, comparing seronegative and seropositive patients for both RF and anti-CCP. No statistically significant differences were observed (data not shown). In fact, no significant differences were observed in seropositive patients compared to seronegative cases, particularly in the early stages of the disease. In addition, a correlation analysis was performed to investigate the possible relationship between the expression levels of all genes in the study, not only in PBMC from all patient groups, but also on isolated B cells from patients with established RA. Nevertheless, no significant differences could be observed (data not shown). Further, to confirm whether the changes in gene expression results of all groups were affected by age, a correlation analysis was performed between age and relative gene expression results for all genes in all groups, but no statistically significant differences were observed (data not shown). To test whether gene expression results were directly related to clinical inflammation markers, a correlation study was performed between the individual relative gene expression values of all genes and the DAS28 and ESR values, but no significant results were detected (supplementary figure available from the author on request).

DISCUSSION

Differences in expression of BAFF, BAFF receptors, and genes related to B cell and immune system activation were found between patients with early RA and patients with established RA.

BAFF is a fundamental B cell survival factor essential for B cell proliferation and activation[12]. BAFF binds to 3 receptors: BAFF-R, BCMA, and TACI, but the precise mechanisms that control expression of all 3 BAFF receptors are currently unclear. Disturbances in BAFF receptor expression occur in several pathologies[13,14,15,16,17] including RA, where it has been demonstrated an increase in both BAFF and BAFF-R mRNA expression in RA synovial tissue[18].

We found that BAFF-R was significantly increased in later stages of RA (in both ERA and RA) and that TACI expression was elevated in all patients from the first weeks of RA development. These observations suggest that disturbances in TACI expression occur from very early in the onset of RA and that BAFF-R expression increases with progression of RA. Further, BAFF serum and gene expression levels were highest in patients with VERA and decreased once disease was established.

Studies with knockout mice have demonstrated that B cell proliferation depends on either BAFF-R or TACI signaling[19]. BAFF-R signaling can result not only in survival enhancement[20,21], but also in B cell maturation[22], while TACI triggering can stimulate isotype switching and plasma cell differentiation[23]. The functional activity of TACI is ambiguous, however[24,25]. Indeed, TACI provides positive signals driving T-independent B cell response[26] and survival of activated B cells and plasmablasts[23,27], but also delivers negative signals suppressing B cell activation[28,29].

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Previous results from our group demonstrated that A proliferation-inducing ligand (APRIL) levels are increased not only in serum from patients with VERA, but also in synovial fluid from those with established RA[4]. TACI binds APRIL and BAFF equally with high affinity[30] and serves as the only receptor for BAFF/APRIL heterotrimers[31]. Because the class-switch recombination (CSR) process depends on APRIL signaling by means of TACI[32], the maintenance of high levels of TACI expression in patients with VERA, ERA, and RA could contribute to the development of CSR and autoantibody production. During the first weeks of RA onset, the elevated BAFF serum levels could negatively regulate BAFF-R expression on B cells and simultaneously upregulate TACI[29]. The induction of TACI expression on activated B cells could serve as a danger signal, reflecting the need to avoid the potential harmful effects of prolonged activation of B cells through BAFF-BAFF-R interaction[20,33]. It has been reported that upregulation of TACI expression serves as a negative feedback against excessive B cell activation mediated by BAFF-R and CD40[29]. Nevertheless, the role of TACI remains unclear and further studies on TACI function are required.

BAFF serum levels detected in patients with VERA and established RA are in agreement with our previous observations[4]. The increased BAFF serum concentration detected in VERA can either contribute to RA development or be produced as a consequence of immune system activation. The small discrepancies observed in BAFF serum levels and gene expression results when comparing VERA to ERA may reflect differences in posttranscriptional processing affecting BAFF protein production, or differences in protein consumption. Nevertheless, BAFF gene expression in VERA tended to be higher than in ERA, similarly to circulating levels of BAFF.

BAFF is produced and secreted mainly by monocytes, activated macrophages, and neutrophils[34,35]. During the disease course, at very early stages cells from the innate immune system such as neutrophils are more activated and have more delayed apoptosis than at later disease stages[36,37]. Thus it is plausible that at earlier stages of RA, activated neutrophils can synthesize higher BAFF mRNA levels and secrete higher amounts of BAFF protein than in the established phase of the disease[34]. Therefore, the exclusion of neutrophils in our present study affects the results obtained for BAFF at the gene level but not at the protein level.

In patients with established RA, normal BAFF mRNA levels have been demonstrated in peripheral blood leukocytes, as well as normal BAFF serum concentrations[13,38]. It is possible that a good clinical response to MTX therapy in our established RA cohort, supported by the decrease in DAS28, ESR, and CRP values, is associated with a decline in BAFF serum and gene expression levels, as suggested for tumor necrosis factor antagonist treatment[39]. Moreover, BAFF overproduction in established RA may be focused more on the affected arthritic joints[13,40].

The results obtained from isolated B cells and PBMC were coincident for the majority of the genes analyzed. Nevertheless, the median levels of relative gene expression detected on isolated B cells were lower in comparison with PBMC. This is probably due to the different proportions of B cells and RNA concentrations present in PBMC and purified B cell samples used in the 2 independent experiments. It has been shown that the percentages of B cells and monocytes in PBMC were positively correlated with genes overexpressed in patients with RA, while the frequency of T cells was associated with genes with a reduced expression[7]. In addition, we have demonstrated that patients with VERA have reduced numbers of pre-switch memory B cells in circulation[3], suggesting that shifts in B cell subsets might also affect the differences observed. Further, differences in receptor expression by individual cells might also affect the observed results. Indeed, BAFF-R is predominantly expressed by B cells[41], although some studies have demonstrated that this receptor is also expressed on a subset of T cells, particularly central and effector-memory type T cells[42]. Some reports have also described that TACI is expressed on activated T cells[43,44]. Nevertheless, while BAFF, BAFF-R, and TACI signaling is clearly relevant for B cell development and activation, there is no consensus on their importance for T cell survival, and further study is required.

The Pax5 gene codifies a transcription factor also known as B cell-specific activation protein, which is essential for B cell commitment as well as B cell development[45]. The increased Pax5 gene expression levels observed in patients with RA in comparison with EA reinforce the relevance of B cells in RA progression compared to other forms of arthritis.

AID and Blimp-1 gene expression levels were within normal ranges in peripheral blood leukocytes in all our patient groups in comparison with healthy controls. Because CSR and somatic hypermutation, processes that both require the action of AID[46], occur during germinal center (GC) responses in organized lymphoid tissues, AID expression in early and established RA is probably higher in B cells infiltrating RA synovium[47] and secondary lymphoid organs[48] than in circulating B cells. Also, Blimp-1 genetic variants have recently been associated with risk of RA[49]. Blimp-1 is expressed in antibody-secreting cells from humans and mice, but is absent from earlier stages of B cell ontogeny[50]. Studies have demonstrated that patients with RA have low circulating levels of plasmablasts (< 3%), similar to healthy controls[3], but have high infiltration of plasma cells in the synovial membrane[51,52]. Thus, the absence of differences in circulating Blimp-1 mRNA levels could be because only a minority of plasma cells are present in circulation, contrary to what is observed in RA joints. In addition, the expression

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of this gene initiates during terminal differentiation of plasma cells[53] during germinal center reactions, which can further justify the results that were obtained. Further, it has been demonstrated that TACI, in contrast to BAFF-R signaling, inhibits plasma cell generation and IgG class switching by reducing Blimp-1 and AID expression[29]. Therefore, with progression of RA, the increased levels of BAFF-R and TACI and the competition for BAFF signaling will determine the fate of plasma cells and antibody production.

CXCR5 and its ligand, CXCL13, involved in B cell chemotaxis, are upregulated in RA synovium[54], suggesting a local role in B cell recruitment toward the synovial membrane. The increased CXCR5 mRNA levels observed in patients with established RA (in both PBMC and isolated B cells) support active B cell trafficking to the synovial membrane and/or secondary lymphoid organs as the disease progresses.

Reports have described increased serum and synovial fluid β_2m levels in RA[55,56] supportive of an ongoing inflammatory process. Similarly, in our study the elevated gene expression levels of β_2m observed in patients with VERA, ERA, and EA indicate early immune system activation not only in the diagnosis of RA, but also in other forms of arthritis. Patients with established RA who are under treatment with immunosuppressive agents such as MTX have a reduction in β_2m gene expression levels, probably owing to the effects of the downregulation of immune responses. Additionally, the absence of variations in Bcl-2 expression in patients with RA in comparison with controls suggests that this gene does not play a significant role in RA pathogenesis[57].

Because the pattern of gene expression was similar in patients with VERA and ERA and no significant differences were detected in the clinical measures (RF and anti-CCP positivity, CRP, ESR, or DAS28 score) or in markers of inflammation (β_2m) between these groups, our observations further corroborate that the expression of B cell-related genes from patients with untreated VERA (< 6 weeks of disease duration) is relatively constant during the first year of RA progression without treatment, reflecting an ongoing active disease state. As RA becomes chronic and treatment is initiated, changes occur in gene expression levels. Treatment with immunosuppressive drugs such as MTX can affect gene expression results[58,59]. Moreover, it should be noted that the interpatient variability associated with the independent groups of patients analyzed, corresponding to different stages of RA development, might affect the results we obtained. It is important to consider that our results reflect an active inflammation supported by the high disease activity in all patient groups, which can affect gene expression, as demonstrated[7,58,59,60,61,62].

Disturbances in the expression of B cell-related survival and activation genes occur in the very early phase of RA. Increased BAFF serum and mRNA levels early in RA development can contribute to the persistence of autoreactive B cells; together with the elevated expression of TACI, this reinforces the importance of earlier introduction of therapies targeting not only B cells directly (anti-CD20) but also BAFF receptors or its ligands[63].

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