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Peripheral Blood B cell Subsets and BAFF/APRIL Levels and their Receptors are Disturbed in Rheumatoid Arthritis but not in Ankylosing Spondylitis

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Abstract

Background: To evaluate the distribution of circulating B cell subsets and their expression of BAFF/APRIL receptors (BAFF-R, TACI and BCMA) as well as circulating levels of BAFF and APRIL in patients with rheumatoid arthritis (RA) or ankylosing spondylitis (AS) compared to healthy controls (HC).

Methods: 59 patients with RA, 61 patients with AS and 61 HC were evaluated. All patients were receiving traditional treatments and had not received prior biological treatment. Peripheral blood B cell subsets were assessed using multicolor flow cytometry using CD27, CD38 and IgD staining. Expression of BAFF-R, TACI and BCMA was analyzed on each cell subset.

Results: Distribution of peripheral B cells subsets was disturbed in RA compared to HC, with a decreased proportion of naïve and transitional B cells ($p<0.005$), whereas B cell subsets were comparable between AS and HC. Circulating BAFF did not differ between the three groups, while the ratio of BAFF/B cell number was significantly higher in RA compared to HC ($p<0.001$). Circulating APRIL levels were increased in RA compared to HC ($p<0.001$). Circulating BAFF and APRIL, and BAFF/B cell ratio did not differ between AS and HC. We also observed increased expression of BCMA, but not BAFF-R in RA, on both naïve and memory B cell subsets (post germinal center) ($p<0.005$), whereas TACI expression was decreased on memory B cells ($p=0.001$). The expression of BAFF/APRIL receptors did not differ between AS and HC.

Conclusion: Disturbances in B cell homeostasis in RA may promote B cell survival and deregulation, favoring the emergence of autoimmune B cells. Conversely, B cell homeostasis is not disrupted in AS.

Keywords: B cells; BAFF; APRIL; Autoimmune diseases

Introduction

The involvement of B cells in systemic autoimmune diseases has emerged as a new concept over the past ten years, leading to new avenues for innovative therapeutic strategies. In fact, B cells play a number of critical roles in inducing and perpetuating autoimmune reactions. They exert different functions during the immune response, including presentation of antigens, release of cytokines, cooperation with and activation of T cells, production of auto-antibodies and they may act also as regulatory B cells [1]. Thus, there is accumulating evidence supporting an active contribution of B cells to different autoimmune diseases, such as systemic lupus erythematosus (SLE), primary Sjögren's syndrome (pSS) and rheumatoid arthritis (RA) [2-4]. The importance of B cells in RA has been highlighted by the success of B cell-targeting therapy using the anti-CD20 monoclonal antibody rituximab. In parallel to this B cell-specific biologic agent, other anti-B cell therapies are currently under development, especially treatments targeting cytokines implicated in B cell survival and/or differentiation [5].

B cell activation and maturation are under the control of soluble and membrane-bound B cell-activating factors that belong to the tumor necrosis factor (TNF) superfamily: the cytokine BAFF (B cell Activating Factor, also called BLys) has emerged as a crucial factor that modulates B cell survival, activation, maturation, tolerance and homeostasis [6-8]. APRIL (A Proliferation-Inducing Ligand) is another member of this superfamily that has similar functions to BAFF [6,7]. BAFF and APRIL are produced by monocytes/macrophages and dendritic cells, and to a lesser extent by T lymphocytes. Excessive BAFF production has been described in several autoimmune diseases, mainly SLE and pSS, but also RA [9-11]. Thus, elevated BAFF levels contribute to break B cell tolerance, and therefore, to promoting B cell autoimmunity. Elevated serum BAFF concentrations described in systemic autoimmune diseases have prompted the development of anti-BAFF therapy in SLE [5,12]. BAFF and APRIL share two receptors, namely TACI (Transmembrane Activator and Cyclophilin ligand Interactor) and BCMA (B Cell Maturation Antigen), while BAFF also binds to a third receptor, i.e. BAFF-R (BAFF Receptor) [13]. Abnormal expression of

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these receptors on B cells has been described in autoimmune diseases, suggesting that a disrupted BAFF/APRIL factor and receptor system may compromise B cell homeostasis [12]. Moreover, the BAFF to B cell ratio has been shown to be elevated and critical for autoreactive B cell survival in murine models [14,15].

In parallel to these well described autoantibody-productive autoimmune diseases, spondyloarthritis (SpA) is a group of disorders classified as chronic inflammatory rheumatic diseases, with ankylosing spondylitis (AS) being the prototype. These diseases mainly affect the sacroiliac joints and the spine, with the progressive development of spine ossifications and ankylosis. There is no identified autoantibody in this disease group at present. However, the presence of B cells has been observed on immunohistologic analysis of joints that are involved in AS, namely the facet joints [16]. Moreover, one open-label study suggested the efficacy of the B cell-depleting agent rituximab in patients with active AS disease, especially in anti-TNF-α naïve subjects [17].

Several abnormalities in peripheral B cell homeostasis have been associated with SLE and pSS [2,3,18]. In contrast, limited data are available for the B cell subsets in patients with RA, despite several studies focusing on the modifications of B cell homeostasis induced by B cell-targeted therapy [4,19-21]. In addition, the BAFF/APRIL cytokine and receptor system was not evaluated in these studies. In AS or other SpA, peripheral blood B cell subsets, circulating B cell survival cytokines and B cell expression of their receptors, have never been evaluated.

In this context, the objective of our study was to investigate the expression of the BAFF/APRIL cytokine receptors on the different B cell subsets together with the measurement of circulating BAFF and APRIL cytokines in patients with active RA who are naïve of biological treatments, especially rituximab and anti-TNF-α agents; as well as in patients with active AS.

Patients and Methods

Patients

This was a single-centre, cross-sectional study. Consecutive inpatients or outpatients with RA or AS treated in the department of Rheumatology were included in the study. Two physicians (ET, DW) recruited patients during scheduled consultations or hospitalizations. Patients with RA who fulfilled the 1987 American College of Rheumatology criteria [22] and patients with AS who met the modified New York criteria [23] were included. In the RA group, patients were receiving traditional disease-modifying anti-rheumatic drugs (DMARDs) (methotrexate, leflunomide, sulfasalazine (SLZ) or hydroxychloroquine) and/or low dose corticosteroids (CTC) (<10 mg prednisone daily). In the AS group, patients received non-steroidal anti-inflammatory drugs (NSAIDs) and/or SLZ. No patient was currently receiving, or had previously received biologic treatment (anti-TNF-α, abatacept, tocilizumab, rituximab or anakinra). Clinical characteristics (disease duration, extra articular manifestations) were recorded in each patient group. In the RA group, disease activity and functional impairment were evaluated using the DAS28 score and Health Assessment Questionnaire (HAQ), respectively. For AS, clinical activity was evaluated using the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI), the Ankylosing Spondylitis Disease Activity Score (ASDAS-CRP score) and the Bath Ankylosing Spondylitis Functional Index (BASFI). Erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) levels were used as laboratory parameters to assess inflammation. Positivity for rheumatoid factors, anti-CPP antibodies and HLA-B27 status were also recorded.

Control subjects

The control group consisted of 61 healthy subjects from the hospital staff without inflammatory conditions or treatment.

All patients and healthy controls (HC) gave written informed consent. The study protocol was performed in accordance with the Helsinki declaration and approved by the local ethics committee (*Comité de Protection des Personnes EST-II*, study registered under the number 09/536).

Methods

Flow cytometry analysis of peripheral B cell subsets: Blood samples were obtained from patients and healthy controls using EDTA-containing collection tubes. Absolute numbers of blood $CD19^+$ B and $CD3^+$ T cells were determined by single platform flow cytometry using the TetraCXP® method, Flow-Count fluorospheres, and FC500® cytometer (Beckman Coulter, Villepinte, France) as previously described [24]. Peripheral blood mononuclear cells (PBMC) were then prepared by density gradient centrifugation on Pancoll (Pan-Biotech, Aidenbach, Germany). Surface expression analysis of PBMC was performed by multicolor staining according to standard protocols using the following monoclonal antibodies against: CD19 PC7, clone J3-119 (Beckman Coulter, Villepinte, France), CD27 APC-H7, clone M-T271, IgD FITC, clone IA6-2 (BD Biosciences, Le Pont de Claix, France), CD38 PercP, clone HIT2 (Biolegend, Ozyme, Saint-Quentin, France), BAFFR PE, clone 8A7 (eBioscience, Rennes, France), TACI PE, clone 165604 and BCMA PE both from R&D Systems (Abingdon, UK). Isotype-matched controls were also used. A minimum of 100 000 lymphocytes were analyzed for all samples. Cells were analyzed on a FACS Canto II using DIVA software (BD Biosciences).

BAFF and APRIL measurement: Serum samples were processed from whole blood, stored in aliquots at -80°C and used after thawing for soluble BAFF (R&D Systems) and APRIL (eBiosciences) measurement using commercially available ELISA kits according to the manufacturer's recommendations.

Statistical analysis: Results are expressed as mean ± standard error of the mean (SEM). Due to a lack of normal distribution of the assessed variables, non-parametric tests were used. Statistical analysis between the three groups (RA, AS and HC) involved non-parametric analysis of variance (ANOVA) using the Kruskal-Wallis test. This test was used to compare age, ESR and CRP, B cell subsets, circulating BAFF and APRIL and BAFF/APRIL receptor expression between the three groups. When analysis between these three groups was significant, a post-hoc Dunn test was performed to evaluate intergroup significance (RA *vs* HC or AS *vs* HC). Qualitative data (gender) were analyzed using the Chi-square test. Disease duration between RA and AS was compared using the Mann-Whitney test. A Spearman's *r*-test was used to calculate correlations between markers of disease activity (ESR, CRP and DAS28) and BAFF/APRIL cytokine levels, and between the B cell subsets and markers of disease activity in the RA population. A p value <0.05 was considered statistically significant for the Kruskal-Wallis, Chi square and Mann-Whitney tests. Due to multiple comparisons against the HC group (RA *vs* HC and AS *vs* HC and RA *vs* AS), Bonferroni correction was applied to the *post hoc* Dunn tests and thus, the level of statistical significance was set at $p<0.016$.

Results

Clinical characteristics of the study population

The demographic and clinical characteristics of the patients and

healthy controls (HC) are shown in Table 1. A total of 59 patients with RA, 61 with AS and 60 HC were included over a two-year period. In RA, most of the patients had traditional DMARDs while just over half (56%) had CTC (mean dose: 5.9 ± 2.8 mg daily). In AS patients, treatments given were NSAIDs and only a limited number had SLZ or CTC (8%, mean dose: 8.4 ± 2.8 mg daily). All patients with RA had active disease (as defined by a DAS28 ≥ 3.2) as had patients with AS (defined as a BASDAI ≥ 4 and an ASDAS ≥ 2.1). Patients with RA were older compared to patients with AS or HC (Kruskal-Wallis test: $p<0.0001$; *post hoc* Dunn test RA *vs* HC: $p<0.0001$). As expected, most

	RA	AS	HC	*P* value*	*P* value#
N	59	61	60		
Age (years), mean ± SEM	58 ± 1.6	43.6 ± 1.8	46.6 ± 1.8	<0.0001	RA vs HC: <0.0001
Gender (M/F), no.	12/47	46/15	13/47	**<0.0001	AS vs HC: **<0.0001
Disease duration (years), mean ± SEM	10.2 ± 1.1	11.7 ± 1.2	NA	NS***	
Extra articular disease, no. (%)					
Nodules	5 (8.4)	0	NA		
Uveitis	0	12 (19.6)	NA		
Sicca syndrome	5 (8.4)	0	NA		
Crohn	0	4 (6.5)	NA		
Psoriasis	0	8 (13.1)	NA		
Rheumatoid Factors (%)	76.3	NA	NA		
Anti-CPP antibodies (%)	76.3	NA	NA		
HLA-B27 (%)	NA	85.3	NA		
DAS28, mean ± SEM	3.9 ± 0.2	NA	NA		
HAQ, mean ± SEM	1.14 ± 0.1	NA	NA		
BASDAI, mean ± SEM	NA	5.2 ± 0.3	NA		
BASFI, mean ± SEM	NA	4.2 ± 0.3	NA		
ASDAS, mean ± SEM	NA	3.2 ± 0.1	NA		
Treatments					
MTX, no. (%)	33 (55.9)		NA		
LFM, no. (%)	10 (16.9)		NA		
Other (SLZ, HCQ), no. (%)	12 (20.3)	4 (6.5)	NA		
AINS, no. (%)		44 (72.1)	NA		
CTC, no. (dosage, m ean ± SEM)	33 (5.9 ± 2.8 mg)	5 (8.4 ± 2.3 mg)	NA		
ESR (mm/h), mean ± SEM	26.6 ± 3.3	21.3 ± 2.9	13.5 ± 2.3	<0.05	RA vs HC: 0.0015
CRP (mg/L), mean ± SEM	12.2 ± 2.3	10.4 ± 2.1	5.7 ± 1.6	<0.0001	RA vs HC: 0.001 AS vs HC: <0.0001

M: Male; F: Female; DAS28: Disease Activity Score 28 joints; HAQ: Health Assessment Questionnaire; BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; BASFI: Bath Ankylosing Spondylitis Functional Index; ASDAS: Ankylosing Spondylitis Disease Activity Score; MTX: Methotrexate; LFM: Leflunomide; SLZ: Sulfasalazine; HCQ: Hydroxychloroquine; CTC: Corticosteroids; ESR: Erythrocyte Sedimentation Rate; CRP: C-Reactive Protein
*Kruskal-Wallis test [level of significance was <0.05]
#post hoc Dunn test [level of significance was <0.016]
**Chi square test
***Mann-Whitney test). NA: not applicable

Table 1: Clinical and demographic characteristics of the patients with rheumatoid arthritis (RA), ankylosing spondylitis (AS) and the healthy controls (HC).

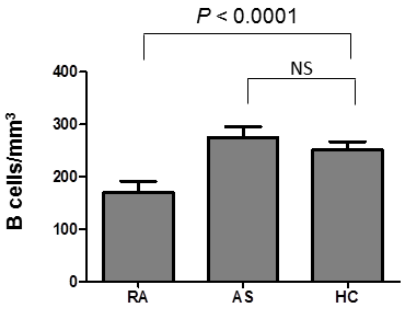

Figure 1: Absolute B cell number in patients with rheumatoid arthritis (RA; N=59), ankylosing spondylitis (AS; N=61) and healthy controls (HC; N=60). Results are mean ± SEM. Statistical analysis was performed using Kruskal-Wallis test for global difference (level of significance: 0.05) and then post hoc Dunn test for comparison between RA and HC and between AS and HC (level of significance: 0.016).

	RA	AS	HC	P value*	P value#
	N = 59	N = 61	N = 60		
CD3+ T cells/ mm^3	1171.5 ± 84.9	1424 ± 66	1572.8 ± 68	0.0007	RA vs HC: <0.0001
CD19+ B cells/ mm^3	171.5 ± 21.6	271.6 ± 20.9	251.6 ± 15.6	<0.0001	RA vs HC: <0.0001
CD19+ B cells (%)	10.9 ± 0.9	14.6 ± 0.7	12.2 ± 0.6	0.0005	RA vs HC: NS
CD27- IgD+ CD38- naïve B cells (%)	52.7 ± 1.7	56.1 ± 1.6	59.3 ± 1.2	0.019	RA vs HC: 0.0025
CD27- IgD- CD38+ transitional B cells (%)	1.9 ± 0.3	2.7 ± 0.3	2.9 ± 0.3	0.0001	RA vs HC: <0.0001
CD27+ IgD+ CD38- memory B cells (%)	10.1 ± 0.7	9.5 ± 0.6	9.8 ± 0.6	NS	
CD27+ IgD+ CD38+ Pre GC B cells (%)	1.3 ± 0.2	1.3 ± 0.2	1.1 ± 0.1	NS	
CD27+ IgD- CD38- Post GC B cells (%)	13.3 ± 1.1	11.6 ± 0.8	11.2 ± 0.6	NS	
CD27++ IgD-CD38++ Plasmablasts (%)	2.6 ± 0.5	1.8 ± 0.3	1.5 ± 0.2	NS	

*Kruskal-Wallis test [level of significance was <0.05]
#Post hoc Dunn test [level of significance was <0.016]

Table 2: Distribution of B lymphocyte subsets in patients with rheumatoid arthritis (RA), ankylosing spondylitis (AS) and healthy controls (HC).

AS patients were men (75%) (Chi square test: p<0.0001). Both ESR and CRP were elevated in RA and AS compared to HC (Kruskal-Wallis tests: $p<0.05$ and $p<0.0001$, respectively).

Peripheral B cells are disturbed in RA but not in AS

T and B lymphocyte counts were significantly different between the three groups (Kruskal-Wallis tests: $p=0.0007$ and $p<0.0001$, respectively), with the decrease in circulating B and T cells significant between RA and HC only (all *post hoc* Dunn tests: $p<0.0001$) (Figure 1). The proportion of B lymphocytes was different between the three groups (Kruskal-Wallis test: $p=0.0005$); the difference was statistically significant between RA and AS (*post hoc* Dunn test: $p<0.001$), but not between RA and HC (post hoc Dunn test: $p>0.016$) (Table 2).

We then examined the relative numbers of naïve B cells and antigen-experienced B cells in patients and HC, according to the expression of CD27, IgD and CD38. Naïve B cells were defined as $CD19^+IgD^+CD27^-CD38^-$, while transitional B cells were $CD19^+IgD^+CD27^-CD38^{++}$. CD27 expression identifies antigen-experienced B cells, including $CD27^+IgD^-CD38^-$ post-germinal center (post-GC), and $CD27^+IgD^+CD38^{++}$ plasmablasts (Figure 2). Pre-germinal center B cells (pre-GC), defined as $CD27^+IgD^+CD38^{++}$, are typically found in tonsils, but have been also described in peripheral blood of SLE patients [25,26]. When analyzing specific B cell subsets, we observed that the proportion of naïve B cells and transitional B cells were significantly different between the three groups (Kruskal-Wallis $p<0.05$ for all tests). This difference was explained by a decreased proportion of these cell subsets in RA compared to HC (*post hoc* Dunn tests RA *vs* HC: % of naïve B cell, $p=0.0025$; and % of transitional B cell, $p<0.0001$), while there was no difference between AS and HC (Table 2). Finally, the percentages of memory, pre GC cells, post GC cells and plasmablasts were similar between the three studied groups (Kruskall Wallis tests: all $p>0.05$) (Table 2).

Serum levels of BAFF, APRIL and BAFF to B cell ratio

We measured the concentration of BAFF and APRIL in the serum of RA and AS patients and HC. No difference in serum BAFF concentrations was observed between the three groups (RA *vs* AS *vs* HC: 1100.5 ± 62.5 *vs* 904.9 ± 29.4 *vs* 939.2 ± 30.3 pg/ml, Kruskal-Wallis test: $p>0.05$). Since there was a decrease in B cells in RA patients, we examined the ratio of circulating BAFF to total B cell count. This ratio was significantly different between the three groups (RA *vs* AS *vs* HC: 24.1 ± 11.5 *vs* 4.6 ± 0.6 *vs* 4.9 ± 0.5 pg/ml/B cell, Kruskal-Wallis test: $p<0.0001$) and this difference was explained by an increased BAFF/B cell ratio in RA compared to HC (*post hoc* Dunn test: $p<0.001$) (Figure 3). APRIL serum concentrations also differed significantly between the three groups (RA *vs* AS *vs* HC: 19116.3 ± 4919 *vs* 5853.5 ± 750.4 *vs* 5699.3 ± 740 pg/ml, Kruskal-Wallis test: $p<0.0001$) due to increased APRIL levels in RA compared to HC (*post hoc* Dunn test: $p<0.001$) (Figure 4).

BAFF and APRIL receptor expression in peripheral B cell subsets

To evaluate the role of BAFF and APRIL, we analyzed the expression of BAFF-R, TACI and BCMA on each peripheral B cell subset. As indicated in Table 3, we observed comparable expression of BAFF-R in the three groups (Kruskall-Wallis tests: all $p>0.05$). Conversely, we

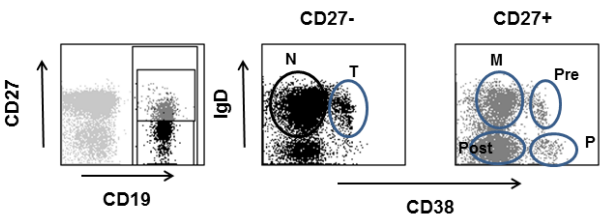

Figure 2: B cell subsets based on the expression of CD27, IgD and CD38. A representative example of the gating strategy used in the phenotype analysis is shown. Mononuclear cells were stained with monoclonal antibodies against CD19, CD27, IgD, CD38 and cells were gated on $CD27^-CD19^+$ and $CD27^+CD19^+$ respectively. Different B cell subsets are indicated as follows: N=naïve, T=transitional, M=memory, pre=pre-GC, post=post-GC and P=plasmablasts.

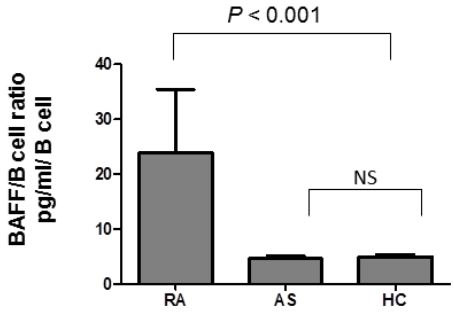

Figure 3: Circulating BAFF levels on B cell number ratio in patients with rheumatoid arthritis (RA; N=59), ankylosing spondylitis (AS; N=61) and healthy controls (HC; N=60). Results are mean ± SEM. Statistical analysis was performed using Kruskal-Wallis test for global difference (level of significance: 0.05) and then post hoc Dunn test for comparison between RA and HC and between AS and HC (level of significance: 0.016).

	RA NS=59	AS NS=61	HC NS=60	P value*	P value#
CD19+ B cells					
BAFF-R	14.4 ± 2	14.2 ± 1.4	12.7 ± 1	NS	
TACI	4.1 ± 0.4	2.6 ± 0.1	2.6 ± 0.1	0.03	RA vs HC: NS; RA vs AS: 0.0075
BCMA	7.6 ± 1	3.4 ± 0.3	3.5 ± 0.3	< 0.0001	RA vs HC: <0.0001
Naïve B cells					
BAFF-R	14.4 ± 1.9	14.5 ± 1.4	13 ± 1	NS	
TACI	2.2 ± 0.3	2 ± 0.1	1.9 ± 0.1	NS	
BCMA	3.7 ± 1	1.8 ± 0.1	1.9 ± 0.1	0.006	RA vs HC: 0.004; RA vs AS: 0.002
Transitional B cells					
BAFF-R	8.3 ± 0.9	10.7 ± 1.1	10.1 ± 0.8	NS	
TACI	19.8 ± 4.5	8.5 ± 2.1	10.3 ± 3.1	0.06	
BCMA	12.9 ± 4.7	5.7 ± 1.3	4.8 ± 1.2	NS	
Memory B cells					
BAFF-R	14.5 ± 1.8	14.6 ± 1.5	13.2 ± 0.9	NS	
TACI	2.6 ± 0.2	2.7 ± 0.2	2.7 ± 0.1	0.01	RA vs HC: 0.001
BCMA	3.3 ± 0.2	2.6 ± 0.1	2.8 ± 0.1	NS	
Pre GC B cells					
BAFF-R	11.9 ± 2.4	12.3 ± 1.3	10.7 ± 0.9	NS	
TACI	16.2 ± 5.1	5.5 ± 0.8	7.9 ± 1.2	NS	
BCMA	5.9 ± 1.1	3.7 ± 0.3	4.3 ± 0.5	NS	
Post GC B cells					
BAFF-R	15.8 ± 2.3	14.3 ± 1.5	13 ± 0.9	NS	
TACI	2.5 ± 0.1	2.6 ± 1.2	2.7 ± 0.1	0.06	
BCMA	3.1 ± 0.1	2.5 ± 0.1	2.5 ± 0.1	0.0006	RA vs HC: 0.001; RA vs AS: < 0.001
Plasmablasts					
BAFF-R	5.9 ± 1.7	4.4 ± 0.6	3.9 ± 0.4	NS	
TACI	6 ± 1.3	4.1 ± 0.4	3.7 ± 0.3	NS	
BCMA	4.4 ± 0.4	3.3 ± 0.2	3.8 ± 0.3	0.06	

Results are expressed as the mean ± SEM of the RFI corresponding to the ratio between the mean fluorescence intensity (MFI) observed with the marker on the B cell subset and the MFI obtained with the isotype control.
*Kruskal- Wallis test [level of significance was <0.05]
#Post hoc Dunn test [level of significance was < 0.016]

Table 3: Expression of the receptors for BAFF and APRIL (BAFF-R, TACI and BCMA) on the different B cell subpopulations in patients with rheumatoid arthritis (RA), ankylosing spondylitis (AS) and healthy controls (HC).

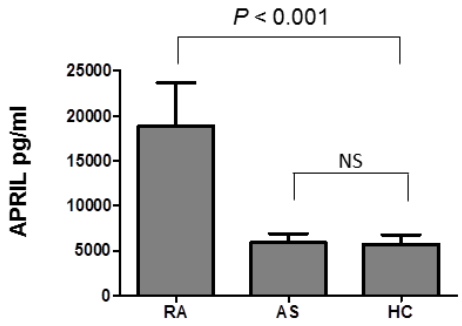

Figure 4: Serum APRIL levels in patients with rheumatoid arthritis (RA; N=59), ankylosing spondylitis (AS; N=61) and healthy controls (HC; N=60). Results are mean ± SEM. Statistical analysis was performed using Kruskal-Wallis test for global difference (level of significance: 0.05) and then post hoc Dunn test for comparison between RA and HC and between AS and HC (level of significance: 0.016).

observed significantly different BCMA expression between the patient groups and HC, on the whole CD19$^+$ B cell population (Kruskal-Wallis test: $p<0.0001$), and on naïve B cells (Kruskal-Wallis test $p=0.006$) and post-GC cells (Kruskal-Wallis test $p=0.0006$). These differences were explained by an increased expression of BCMA in RA compared to HC (*post hoc* Dunn tests ≤ 0.016 for both naïve and post-GC cells). For TACI expression, significant differences were found for the whole CD19$^+$ B cell population (Kruskal-Wallis test $p=0.03$) and for the memory B cells (Kruskal-Wallis test $p=0.01$). For CD19$^+$ B cells, these differences were related to increased expression of TACI in RA compared to AS (but not HC) (*post hoc* Dunn test: $p=0.0075$) while TACI expression on the memory B cells was decreased in RA as compared to HC (*post hoc* Dunn test: p=0.001) (Table 3).

Finally, we examined the relationships between the soluble factors BAFF and APRIL and the different indices of disease activity in RA. A weak correlation was observed between BAFF levels or BAFF/B cell ratio and CRP, without reaching statistical significance (r=0.24, $p=0.07$ and r=0.25, $p=0.06$ respectively). There was no association between the different B cell subsets and biological markers of inflammation or disease activity (DAS28) in RA patients.

Discussion

This study was undertaken to better characterize B cell subset abnormalities in RA and AS patients. In RA patients, changes in the distribution of B cell subpopulations were detected, mainly affecting the proportion of naïve cells, *i.e.* naïve and transitional B cells. In parallel, we did not observe any modification in the proportion of memory B cells (including pre-GC and post-GC B cells) and plasmablast cells. Previous works evaluating the distribution of naïve and memory B cells in RA have yielded conflicting results. Souto-Carneiro et al. reported a low frequency of blood CD27$^+$IgD$^+$ pre-GC memory B cells while CD27$^+$IgD$^-$ post-GC B cells increased with the duration of the disease [19]. Interestingly, in this study, the authors were able to demonstrate the presence of pre-GC memory B cells in the synovium, suggesting that this cellular subset migrates or accumulates within the joints. No assessment of the BAFF/APRIL soluble factors and their receptors on B cells was performed. Sellam et al. found that both naïve and memory B cells, especially CD27$^+$IgD$^-$ post-GC memory cells, were decreased in a large RA patient population participating in a rituximab clinical trial [20]. However, the population included a large number of patients who had received prior anti-TNF-α treatment. The study by Fekete et al. reported less naïve and more CD27$^+$IgD$^-$ post-GC memory B cells, but again, a high proportion of these patients were treated by TNF-α blocking agents at the time of assessment [4]. The discrepancies between these studies may be explained by the patient characteristics (age, disease duration), the treatment received and especially prior biological treatment. Indeed, it has been shown that TNF-α blocking agents may influence B cell subsets. In particular, etanercept, the p75 TNF-α soluble receptor/Ig fusion protein, may impair germinal center formation and decrease the number of CD27$^+$ memory cells [27]. Infliximab, an anti-TNF-α monoclonal antibody, was shown to be associated with an increase in the frequency of CD27$^+$IgD$^+$ pre-GC memory B cells in the peripheral blood of patients with RA [19]. Rituximab also exerts various effects on B cell subsets [21,28]. Peripheral blood B cell subsets are thus sensitive to biological agents. Our main findings in biologic-naïve RA patients are a decrease of total B cells, associated with a decreased proportion of naïve B cells.

Although the equilibrium between naïve and memory B cells was relatively well preserved, our RA patients were characterized by an overall decrease of both T and B cells, as has previously been reported [24]. The lymphopenia affecting T cells in the RA group may be explained by traditional treatments such as methotrexate. It has been suggested that B cell subsets that are decreased in the peripheral blood may have migrated to the target organ, i.e. the synovium [19], or alternatively to lymphoid tissues [21]. In this way, presence of both pre- and post-GC memory B cells has been observed in synovial biopsy samples from RA patients [19]. Another explanation for these B cell subset changes could be the effect of the disease, and accordingly, an association between some B cell subsets (CD27$^+$ memory B cells) and markers of B cell activation (elevated serum Ig levels, serum free light chains or serum BAFF levels) has been reported [20]. However, in our study, we did not observe any relationship between the B cell subsets and the different (clinical or biological) markers of disease activity. Moreover, all the patients assessed in the different studies of B cell subsets were receiving treatment, with at least traditional DMARDs, and/or CTC, as in our study. It is therefore impossible to distinguish between the impact of the disease itself, and that of the treatments (DMARDs and/or CTC) on the B cell subset changes that we observed. Patients affected by early-stage and untreated RA disease may be the ideal population to investigate this further, but this type of population was unfortunately inaccessible for our study.

Another striking finding in our study is a higher BAFF/B cell ratio and elevated serum APRIL level in the RA group. As discussed above, high levels of these soluble factors have been described in patients with RA [29] or very early RA [30] or in other autoimmune diseases [29,31,32]. Since BAFF levels per B cell have been determined in murine models to be critical in autoreactive B cell survival [14,15], and a decrease in B cells characterized patients with RA, it seems more relevant to evaluate the BAFF/B cell ratio rather than circulating BAFF. BAFF is well known as a critical soluble factor for B cell survival and homeostasis [13]. Its role in autoimmunity is now well understood and illustrated by the clinical efficacy of anti-BAFF therapies in SLE [12]. APRIL shares similar functions with BAFF, but the precise biological role of APRIL in immune cell development and regulation requires further clarification [6-8]. The results on serum BAFF levels in our patients with RA did not fit completely with those of previous studies [29,30]. In fact, we observed an increase in BAFF levels in RA patients as compared to AS and HC, although this increase was not significant. These discrepancies may be explained by the patient's characteristics,

the disease duration, the disease activity, and presumably, by the treatment they received. However, the effects of anti-rheumatic drugs on the levels of circulating BAFF are not well understood. It has been described that cytokines, such as IL-10, IFN-α and IFN-γ may increase BAFF expression in various cell types [7,8], while high-dose corticosteroid treatment inhibits its expression [33]. Biological treatments, such as rituximab, induce BAFF, while TNF-α blockade does not affect BAFF levels [34].

We also observed increased expression of the BAFF/APRIL receptors involving BCMA, but not BAFF-R on both naïve and memory B cell subsets, whereas TACI expression was decreased on memory B cells. These receptors (BAFF-R, TACI and BCMA) have been reported to be differently expressed on the B cell subsets in HC: TACI is found predominantly on $CD27^+$ memory B cells, BCMA is highly expressed by plasma cells, plasmablasts and tonsilar germinal center, while BAFF-R is found on all peripheral B cells [6,7]. BAFF-R is considered to be the predominant receptor of B cells, but can only bind to BAFF, with this interaction driving B cell maturation and survival. TACI is a negative regulator for B cell responses, while BCMA is an important effector for humoral immune response, and thus, is expressed during the late stage of B cell differentiation [7]. Taken together, the balance between the high levels of BAFF/B cell ratio (representing active BAFF availability) and circulating APRIL, the increased BCMA expression and a decreased expression of the negative regulator TACI described in this study, strongly support an influence on B cell survival in RA patients. One hypothesis is that in the setting of the reduced B cell number and especially low naïve B cell frequency, an excess of soluble BAFF/APRIL factors and high expression of BAFF/APRIL receptors will promote B cell survival, including autoreactive B cells, and thus autoimmunity, as previously suggested in the context of chronic graft-versus-host-disease after allogeneic hematopoietic cell transplantation [31]. Our results are also in accordance with a previous work showing no significant difference in the expression of BAFF-R between RA patients evaluated before rituximab therapy and HC [35]. Overall, our study has the advantage of performing the analysis of both B cell subsets, B cell survival factors and B cell expression of their cognate receptors. On this basis, abnormalities of B cell homeostasis are observed in RA patients.

In this study, we also evaluated the role of B cells in AS. It is acknowledged that AS and related SpA do not have an autoimmune background, despite evidence showing an active contribution of the immune system. Whether B cells participate in inflammatory responses in AS or not is currently undetermined. In order to determine the involvement of B cells in AS, we evaluated the different B cell subsets and BAFF/APRIL soluble molecules as well as the B cell expression of their receptors in patients with active AS. Again, and to avoid confusing results, we selected biologic-naïve patients. In the AS population, patients were receiving mainly NSAIDs, and thus, our results could not be biased by a treatment (DMARD) effect. In addition, only a minority of these patients had CTC. Our results show neither disturbed B cell subset distribution nor modifications in BAFF/APRIL concentrations or BAFF/APRIL receptor B cell expression. This means that B cell homeostasis is preserved in AS, and likely in SpA. This can be related to the absence of autoantibody production in these diseases. The presence of B cells has been demonstrated in the facet joints of patients with AS [16], while they do not predominate at the main target joints, i.e. the sacroiliac joints [36]. In addition, rituximab has been reported to be clinically effective in patients with AS but only in uncontrolled and open-label studies [17].

In conclusion, our results show clear disturbances in B cell homeostasis, with biased distribution of B cell subsets, high levels of B cell activating factors and changes in the expression of the BAFF/APRIL receptors BCMA and TACI on B cells in RA, but not in AS. These changes in RA may promote B cell survival and B cell deregulation, favoring the emergence of autoimmune B cells. However, whether these abnormalities in B cell distribution, BAFF/B cell ratio and BAFF receptor expression may help to identify responders to B-cell depleting therapy or not remains still controversial. A better understanding of the role played by the B cell populations in the mechanisms leading to autoimmunity may help to better define specific therapeutic approaches targeting B cells and the BAFF/APRIL pathway. On the other hand, B cell homeostasis is not disrupted in AS and our results do not argue in favor of proposing B cell depleting or BAFF-targeted therapy in this group of disorders.

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Conflict of Interest

The authors have no conflict of interest to declare.

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