ZAP-70+ B Cell Subset Influences Response to B Cell Depletion Therapy and Early Repopulation in Rheumatoid Arthritis

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ABSTRACT. ***Objective.*** To define the role of ZAP-70+ B cells (CD19+/ZAP-70+) as a biomarker of response to B cell depletion therapy (BCDT), their relationship with clinical outcome, and their behavior during repopulation of peripheral blood in patients with rheumatoid arthritis (RA).

Methods. Thirty-one patients with RA underwent BCDT and were followed for 12 months. Disease activity was assessed with the European League Against Rheumatism (EULAR) criteria. Cytofluorimetric analysis of peripheral blood B cell subsets at baseline and at 6- and 12-month intervals after BCDT was performed using surface markers (CD45, CD3, CD56, CD19, IgD, CD38, CD27) and intracellular ZAP-70.

Results. A moderate/good EULAR response was achieved in 66.6% of the RA cohort. The baseline percentage of CD19+/ZAP-70+ cells was lower in good responder patients (1.8% ± 1.7%) compared to poor responders (5.6% ± 4.9%; p = 0.02). A decrease of plasmablasts (IgD-CD27+CD38+) and pre-switch memory (IgD+CD27+) B cells occurred after BCDT. Recovery of B cells in peripheral blood after the first course of BCDT was characterized by the reappearance of B cell subtypes that showed a naive, activated phenotype, coupled with a decrease in memory cells. B cells carrying intracytoplasmic ZAP-70 increased significantly from the baseline value of 4.4% ± 4.5% to 12.4% ± 9.2% (p = 0.001) at the 6-month and to 9.4% ± 6.4% (p = 0.002) at the 12-month followup.

Conclusion. Baseline percentage of CD19+/ZAP-70+ cells is associated with the clinical outcome after BCDT in patients with RA. Depletion of plasmablasts and pre-switch memory B cells and increase of CD19+/ZAP-70+ cells are features of the recovery of the B cell pool after BCDT. (First Release Sept 15 2012; J Rheumatol 2012;39:2276–85; doi:10.3899/jrheum.120153)

Key Indexing Terms:

B CELL SUBSETS | ZAP-70 | RHEUMATOID ARTHRITIS
RITUXIMAB | BIOMARKERS | DEPLETION THERAPY

Rheumatoid arthritis (RA) is a chronic inflammatory disease whose course is influenced by the early phase, which represents a crucial period for therapeutic intervention[1,2,3]. Among several pathways involved in the autoimmune homeostasis in RA, the complex deregulation of B lymphocytes has rendered these cells such good therapeutic targets that novel treatment regimens affecting B cells have recently been developed[4].

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Rituximab (RTX) successfully depletes CD20-expressing B cells by directly targeting the intermediate B cell stages. RTX does not affect pre-B cells and long-lived plasma cells[5,6]. CD20+ B cell depletion in RA is complete at 1 month after the start of a single treatment and it is sustained for several months[7]. RTX acts on B cells through different mechanisms such as apoptosis and antibody-dependent and complement-dependent cytotoxicity[8].

There are, however, no confirmatory data on how B cell depletion can exert its clinical effect, since B cell depletion at the synovial level is often incomplete even in good responders[9,10,11]. Despite effective depletion of circulating B cells in nearly all treated patients, a substantial percentage of patients usually do not respond to RTX treatment. Since RTX depletes circulating B cells equally in responders and nonresponders, the mechanism by which the clinical response is achieved is not entirely clear[12].

It has been demonstrated that clinical response to B cell depletion therapy (BCDT) is related to the serological status, with positivity for rheumatoid factor (RF) and/or anti-citrullinated protein antibodies (ACPA) associated with

a better clinical outcome. Further, previous exposure to more than one anti-tumor necrosis factor-α (anti-TNF-α) agent has been found to be related to a poor response[13,14,15].

One of the most challenging issues is the identification of biomarkers for therapeutic outcome. Thus the influence of B cell subsets warrants further investigation.

The cytofluorimetric assay is a useful tool to examine changes induced in the B cell compartment by RTX. Studies investigating the presence and distribution of B cells in peripheral blood and bone marrow before and after BCDT with RTX have shown that naive B cells represent the first B cell subset in the repopulation phase, followed by the memory B cell pool[16,17,18]. There has been increasing interest in molecular or cellular markers that predict which patients could benefit from BCDT.

ZAP-70, a tyrosine kinase constitutively expressed in T cells, is fundamental for their development and activation because the molecule is a key regulator of signal transduction of the T cell receptor, as demonstrated by the severe immunodeficiency status that arises in those individuals who lack ZAP-70[19]. Recent studies have demonstrated that ZAP-70 can be expressed by B cells, particularly in chronic lymphocytic leukemia (CLL), in which ZAP-70 enhances B cell receptor signaling. ZAP-70 was found to be a crucial prognostic factor in CLL[20]. Recent research by our group focused on the expression of ZAP-70 by B cells in both synovial fluid and tissue from patients with RA[21,22]. In synovial fluid, the presence of ZAP-70+ B cells showed a correlation with expression of autoantibodies and levels of inflammatory cytokines such as interleukin 6 (IL-6) and B cell activating factor (BAFF). In the synovial compartment, the presence of ZAP-70 was demonstrated in B cells exhibiting a memory/plasmablast phenotype and characterized an aggregate pattern of synovitis.

We focused on the presence of circulating ZAP-70-positive B cells at baseline and after BCDT to define the possible role of these cells as biomarkers of response to treatment. The secondary aim was to assess whether this B cell subtype might be a marker of regeneration–repopulation after the BCDT.

MATERIALS AND METHODS

Patients. Thirty-one patients with RA were treated with BCDT in the Division of Rheumatology, Catholic University of the Sacred Heart, between 2008 and 2011. All patients fulfilled the 1987 American College of Rheumatology classification criteria[23]. They all had active disease and had previous poor response to traditional disease-modifying antirheumatic drugs (DMARD) and/or to anti-TNF-α therapy or presented comorbidities contraindicating such therapy. The duration of followup ranged from 6 to 12 months and the number of patients with adequate data are indicated as applicable. A group of healthy sex- and age-matched controls were also enrolled.

Therapy. All patients received a first course of 2 infusions of intravenous RTX 1 g two weeks apart. Steroid therapy was allowed at the maximal dose of 7.5 mg prednisone daily. Patients received RTX with MTX at doses of up to 20 mg/week or with other DMARD (leflunomide 20 mg/day or chloroquine 4 mg/kg/day) when intolerant to MTX. After 6 months, patients were stratified into 2 groups: those with low residual disease activity or good responders, i.e., Disease Activity Score (DAS) < 2.4 coupled with a decrease of ≥ 1.2 from baseline; and those with high residual disease activity or poor responders, i.e., DAS ≥ 2.4 and a decrease ≤ 1.2 from baseline value. Subsequent courses of RTX were considered after at least 6 months in those patients who presented residual disease activity or exhibited a clinical relapse after the initial response, i.e., DAS worsening by 0.6. In the former situation, retreatment was administered using a 2-infusion scheme, i.e., 500 mg two weeks apart, while in the latter, RTX was administered using the hematological regimen (375 mg/m^2 on Days 1, 7, 14, and 21).

Clinical outcomes. Demographic and clinical data, Health Assessment Questionnaire (HAQ) responses, and DAS findings were recorded at baseline (T0) and at 3-month intervals for all patients. Treatment efficacy was determined at 6 months (T6) and 12 months (T12) after the first RTX infusion according to the European League Against Rheumatism (EULAR) response criteria. The EULAR response criteria were used to classify patients as good or moderate responders or nonresponders[24].

Laboratory assessments. Erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) levels, immunoglobulin levels, white blood cell counts, and total number of peripheral lymphocytes and lymphocyte subpopulations were evaluated at baseline and at 3, 6, and 12-month followup intervals for each patient.

Each patient was tested for RF isotypes (IgA and IgM RF), ACPA, and anti-vimentin antibodies (MCV) at baseline and at 6-month intervals. ACPA were measured using an ELISA method (Axis-Shield Diagnostics) with positive level defined as ≥ 5 U/ml. Anti-MCV antibodies and RF IgA and IgM isotypes were measured by ELISA (Orgentec Diagnostika GmbH) and considered positive above a cutoff value of 20 U/ml as suggested by the manufacturer.

Plasma levels of IL-6 (Becton Dickinson Bioscience) and BAFF were measured by ELISA (R&D Systems). The sensitivity of the test was 2.2 pg/ml for IL-6 and 3.8 pg/ml for BAFF.

B cell immunophenotyping. Peripheral blood samples were collected by venipuncture into EDTA tubes at baseline and every 6 months thereafter and were analyzed by flow cytometry for distribution of circulating B cell subsets. Immunofluorescence labeling for flow cytometry was performed by incubating 100 μl peripheral blood with anti-human monoclonal antibodies. Cells were stained with anti-human antibodies specific for CD45 [antigen-presenting cell (APC)-A750], CD19 (APC-700), CD3 (ECD), CD56 (ECD), CD38 (PC5 or APC), CD5 (APC), CD23 (APC), CD27 (PC7), and FITC-conjugated IgD (Beckman Coulter). After 20 min incubation, samples were fixed with 200 μl Reagent 1 (Beckman Coulter) for 15 min at room temperature in the dark. After washing, the pellet was incubated with 200 μl Reagent 2 (Beckman Coulter) for 15 min at room temperature in the dark. Samples were then incubated with PE-conjugated ZAP-70-specific antibody (clone SBZAP; Beckman Coulter) for 30 min at room temperature in the dark. After staining, the cells were washed and immediately analyzed on a compensated 8-color Navios flow cytometer and data were elaborated with the Kaluza program (Beckman Coulter). Lymphocytes were gated on the basis of forward and side-scatter properties, confirmed through CD45 staining. B cell subsets were evaluated through the expression of surface B cell markers according to IgD/CD38 and IgD/CD27 classifications (Appendix 1)[16,25]. Expression of ZAP-70 in CD19+ cells (B cells) was measured according to the gating strategy published by Crespo, *et al*[26]. The first gate was set on lymphocytes defined by forward and side-scatter characteristics and expression of CD45. B cells were defined as CD56- and CD3-negative and CD19-positive cells. At least 1000 events referred to as CD19-positive cells were acquired for each sample. All samples were then analyzed with a gate on CD3+CD56+ cells to ensure that at least 10,000 T cells were acquired. The cutoff for ZAP-70 expression on B cells was set at 98% of ZAP-70 positivity of T and natural killer cells (Appendix 1)[21,26].

Statistical analysis. Data were analyzed using SPSS v. 17.0 for Windows and GraphPad Prism 5.0 software. Categorical and quantitative variables were described as frequencies and percentages, means and SD. The Kruskal-Wallis test was used to evaluate differences in continuous variables among groups. Differences between 2 groups were tested by Mann-Whitney U test. Categorical variables were analyzed by chi-square test or Fisher's exact test. The Wilcoxon test was used to compare B cell subpopulations and soluble biomarkers during followup in the RA cohort. The Spearman rank correlation test was used to evaluate the relationship between B cell subpopulations and clinical and immunological characteristics.

A receiver-operating curve (ROC) analysis of continuous measures (i.e., B cell subsets at baseline) significantly related to good EULAR response 6 months after BCDT in RA patients was performed to obtain relevant thresholds allowing prediction of response to therapy at the individual level. The nonparametric ROC plot uses all the data, makes no parametric assumptions, and provides unbiased estimates of sensitivity and specificity. Calculation of area under the curve (AUC) provides a convenient single number. AUC values ≥ 0.600 or ≤ 0.400 were considered to be discriminative. The optimal cutoff point was determined to yield the maximum corresponding sensitivity and specificity. The variables related to response to therapy with $p \leq 0.10$ in univariate analysis were entered into a multivariate logistic regression model in which good EULAR response 6 months after BCDT was the variable to be explained. Results are expressed as OR and 95% CI. Statistical significance was defined as $p < 0.05$.

RESULTS

Baseline characteristics of the 31 patients with RA, the group previously treated with anti-TNF and the group previously treated only with DMARD, and 30 controls are shown in Table 1. At baseline, 22 (71.0%) of 31 patients had a DAS > 3.7. At study entry, 25 patients (80.6%) were taking a daily low dose of corticosteroids (7.5 mg prednisone or dose equivalent), while 22 (71.0%) were in stable treatment with methotrexate 10–20 mg/week. Nineteen patients (61.3%) had previously been treated with TNF-α blockers. The main reason for withdrawal of anti-TNF therapy was lack of efficacy in 15 patients (78.9%), while 4 patients (21.1%) stopped anti-TNF treatment because of side effects. Anti-TNF and DMARD-only previous treatment groups of RA patients had similar baseline demographic and immunological characteristics, except for disease duration, which was longer in the anti-TNF treatment group ($p = 0.04$).

At the time of the analysis, all patients had already reached the 6-month followup, whereas only 25 patients had completed the 12-month followup.

Inflammatory biomarkers in patients with RA and controls. At baseline, patients with RA showed higher plasma levels of IL-6 compared to controls (22.8 ± 37.5 pg/ml vs 0.91 ± 0.89 pg/ml; $p < 0.001$). No differences were seen in plasma BAFF levels between patients with RA and controls at baseline (592.24 ± 437.19 pg/ml vs 430.99 ± 174.88 pg/ml; $p = 0.32$); or in baseline plasma BAFF and IL-6 levels between patients previously treated with anti-TNF or DMARD [BAFF: 546.60 ± 323.28 pg/ml in anti-TNF-treated vs 662.78 ± 582.95 pg/ml in DMARD-treated patients ($p = 0.93$); IL-6: 32.49 ± 45.57 pg/ml in anti-TNF-treated vs 7.80 ± 8.12 pg/ml in DMARD-treated patients ($p = 0.23$)].

Peripheral blood distribution of B cell subsets in patients with RA and controls at baseline. At study entry, patients with RA showed a significantly higher white blood cell count compared to healthy controls (7645.5 ± 2801.9 vs 6047.3 ± 1288.7; $p = 0.04$). At study entry, no significant differences were found in total lymphocytes and B cells (percentage or absolute cell number) between patients with

Table 1. Baseline demographic, clinical, and immunological characteristics of patients with RA (previously treated with DMARD and anti-TNF) who had received B cell depletion therapy. Values are mean ± SD unless otherwise indicated.

Characteristics	RA Patients, n = 31	Anti-TNF Group, n = 19	DMARD Group, n = 12	p
Age, yrs	56.35 ± 14.2	54.1 ± 14.0	59.9 ± 14.4	0.35
Females, n (%)	28 (90.3)	17 (89.5)	11 (91.7)	0.84
Disease duration, yrs	12.6 ± 8.3	14.9 ± 8.7	9.0 ± 6.3	0.04
ESR, mm/h	41.7 ± 19.2	41.3 ± 18.7	42.4 ± 21.7	0.92
CRP, mg/l	21.3 ± 37.5	26.6 ± 47.0	13.3 ± 10.3	0.44
DAS	4.0 ± 1.3	4.59 ± 1.30	3.86 ± 1.24	0.92
HAQ	1.7 ± 0.9	1.59 ± 0.85	1.90 ± 0.86	0.29
MTX (10–20 mg/wk), n (%)	22 (71.0)	13 (68.4)	9 (75.0)	0.69
Low-dose corticosteroids (7.5 mg/day), n (%)	25 (80.6)	16 (84.2)	9 (75.0)	0.53
Previous anti-TNF-α therapy, n (%)	19 (61.3)	—	—	—
ACPA > 5 U/ml, n (%)	26 (83.9)	16 (84.2)	10 (83.3)	0.95
RF IgA > 20 U/ml, n (%)	9 (29.0)	6 (31.6)	3 (25.0)	0.69
RF IgM > 20 U/ml, (%)	19 (61.3)	11 (57.9)	8 (66.7)	0.63
Anti-MCV > 20 U/ml, (%)	24/28 (85.7)	14 (82.4)	10 (90.9)	0.53
Seronegative patients*, n (%)	2/31 (6.5)	1 (5.3)	1 (8.3)	1.00

* ACPA, IgA and IgM RF, RT, anti-MCV. TNF: tumor necrosis factor; DMARD: disease-modifying antirheumatic drugs; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; DAS: Disease Activity Score; HAQ: Health Assessment Questionnaire; ACPA: anticitrullinated protein antibodies; MTX: methotrexate; RF: rheumatoid factor; MCV: modified citrullinated vimentin.

RA and controls. Moreover, the distribution of B cell subsets was similar between the 2 groups (Table 2).

Anti-TNF-treated and DMARD-treated RA groups had similar distributions of B cell subsets at baseline, except for the percentage of CD19+/IgD+CD27+ and CD19+/CD5+ cells, which were higher in anti-TNF-treated compared to DMARD-treated patients [CD19+/IgD+CD27+ cells: 8.2% ± 5.9% vs 3.7% ± 2.5% (p = 0.02) and CD19+/CD5+ cells: 11.0% ± 6.1% vs 4.2% ± 2.1% (p = 0.03); Appendix 2].

In the patients with RA, the percentages of CD19-positive cells at baseline were inversely correlated with the percentages of post-switched and double-negative memory B cells (IgD–CD27+: r = –0.48, p = 0.01; IgD–CD27–: r = –0.42, p = 0.02) and with the percentage of CD19+/ZAP-70+ B cells. Moreover, the percentage of ZAP-70-positive B cells was directly correlated with the percentage of memory B cells (eBm5+Bm5; Figure 1A, 1B).

Considering the inflammatory milieu, plasma levels of IL-6 at baseline were inversely correlated with the percentage of CD19-positive cells (r = –0.42, p = 0.03) and directly correlated with CD19+/ZAP-70+ cells (Figure 1C). In contrast, plasma BAFF levels at baseline were inversely correlated with memory Bm5 cells (r = –0.38, p = 0.05) and with plasmablasts (r = –0.41, p = 0.03).

Baseline B cell subset distributions and EULAR response to BCDT. At 6-month followup, 10 patients with RA (33.3%) achieved a good EULAR response, and 10 (33.3%) achieved a moderate EULAR response. Nine (36.0%) of the 25 patients had a good EULAR response after completing the 12-month followup.

No differences were seen in the response rates (moderate and good EULAR response) between patients with RA previously treated with anti-TNF plus DMARD (61.9% with moderate and 28.6% with good EULAR response) and those treated only with DMARD [70.0% with moderate (p = 1.00) and 40.0% with good EULAR response (p = 0.69)].

The patients with detectable B cells were 68.0% at 6-month and 64.0% at 12-month followup. Baseline B cell subset distribution in the patients with RA stratified according to either good or moderate EULAR response at the 6-month followup is illustrated in Figure 2 and Appendix 3.

We further observed that the percentage of baseline naive activated B cells (Bm2) was higher in patients with RA who were responders to BCDT at the 6-month followup compared to the nonresponders (p = 0.03); this was confirmed by a higher value of Bm2+Bm2'/eBm5+Bm5 ratio (p = 0.01). In particular, the patients with RA with good EULAR response were characterized by a similar percentage of baseline Bm2 cells compared to moderate responders (46.5% ± 14.2% in good vs 40.6% ± 20.7% in moderate responders; p = 0.36), but a higher percentage compared to nonresponders (25.7% ± 12.7%; p = 0.01 vs good responders). Moreover, the Bm2+Bm2'/eBm5+Bm5 ratio was higher in good (2.74 ± 2.36) and moderate (2.94 ± 3.29) EULAR responders compared to nonresponders (0.70 ± 1.47; p = 0.02 vs good responders and p = 0.03 vs moderate responders) at the 6-month followup after BCDT. The other B cell subsets were found to be similar when comparing patients with different BCDT responses (Figure 2).

At the 6-month followup, the patients with RA who were good responders were characterized by a lower percentage of baseline CD19+/ZAP-70+ cells compared to moderate responders (1.8% ± 1.7% vs 5.6% ± 4.2%, respectively; p = 0.02) and poor responders (5.7% ± 5.6%; p = 0.11; Figure 2H). Considering 5.0% as the cutoff value for CD19+/ZAP-70+ (mean + 2 SD of control values), RA subjects with baseline CD19+/ZAP-70+ percentage < 5.0% were likely to achieve a good EULAR response (44.4%) compared to patients with baseline CD19+/ZAP-70+ percentage ≥ 5.0% (9.1%; p = 0.05).

The patients with RA who had good EULAR response to the BCDT at the 6-month followup did not differ from healthy controls for baseline distribution of B cell subsets. Poor responders, however, were characterized by a significantly lower percentage of Bm2+Bm2' (33.9% ± 18.2%) compared to healthy subjects (43.0% ± 12.5%; p = 0.002).

For each of the variables significantly associated with good EULAR response, cutoff values were evaluated using the ROC analysis (Appendix 4). The cutoff values were 26.0% for Bm2 and 1.08 for the Bm2+Bm2'/eBm5+Bm5 ratio. Good EULAR response at the 6-month followup was associated in the univariate analysis to Bm2 > 26.0% (p =

Table 2. B cell subset distribution in patients with RA before the B cell depletion therapy compared to healthy controls. Values are mean ± SD.

	Controls, n = 30	RA Patients, n = 31	p
White blood cells	6047.3 ± 1288.7	7645.5 ± 2801.9	0.04
Lymphocytes, %	33.2 ± 8.1	33.2 ± 13.4	0.74
Lymphocytes/mm^3	1932.9 ± 384.5	2391.8 ± 1059.6	0.14
CD19+ B cells, %	9.6 ± 2.9	7.7 ± 4.6	0.08
CD19+ B cells, n	181.1 ± 71.2	178.7 ± 134.7	0.61
Bm1, %	23.3 ± 10.4	24.9 ± 15.8	0.85
Bm2, %	41.2 ± 11.9	37.2 ± 18.0	0.25
Bm2′, %	1.7 ± 1.1	1.5 ± 2.0	0.06
Bm2+Bm2′, %	43.0 ± 12.5	38.8 ± 18.3	0.24
Bm3–4, %	0.7 ± 0.4	0.8 ± 1.2	0.13
eBm5, %	16.0 ± 7.1	15.2 ± 11.0	0.40
Bm5, %	15.1 ± 6.5	17.0 ± 14.7	0.83
eBm5+Bm5, %	31.1 ± 12.1	31.3 ± 18.2	0.64
Ratio Bm2+Bm2/eBm5+Bm5	1.6 ± 0.9	2.1 ± 2.6	0.19
CD19+/IgD+CD27–	60.8 ± 11.8	57.3 ± 17.6	0.40
CD19+/IgD+CD27+	6.7 ± 3.4	6.7 ± 5.4	0.36
CD19+/IgD–CD27+	16.3 ± 7.0	12.9 ± 17.4	0.26
CD19+/IgD–CD27–	16.2 ± 9.1	12.4 ± 5.3	0.16
CD19+/CD27+CD38+	8.0 ± 3.5	7.3 ± 5.5	0.27
CD19+/ZAP-70+	2.2 ± 1.4	4.4 ± 4.5	0.37
CD19+/CD5+, %	13.8 ± 8.5	9.1 ± 6.1	0.11

RA: rheumatoid arthritis.

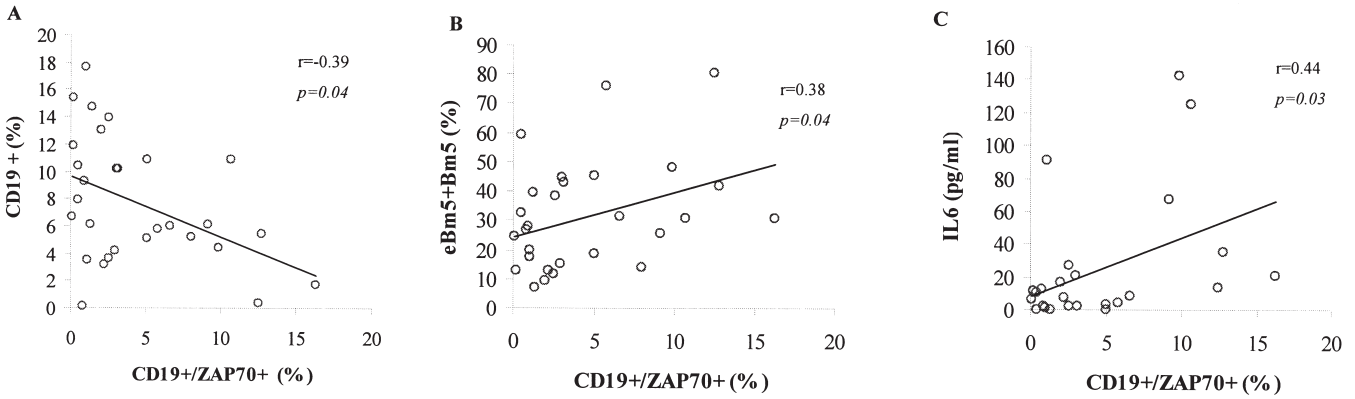

Figure 1. Correlations between baseline B cell subsets and plasma levels of interleukin (IL) 6 before B cell depletion therapy.

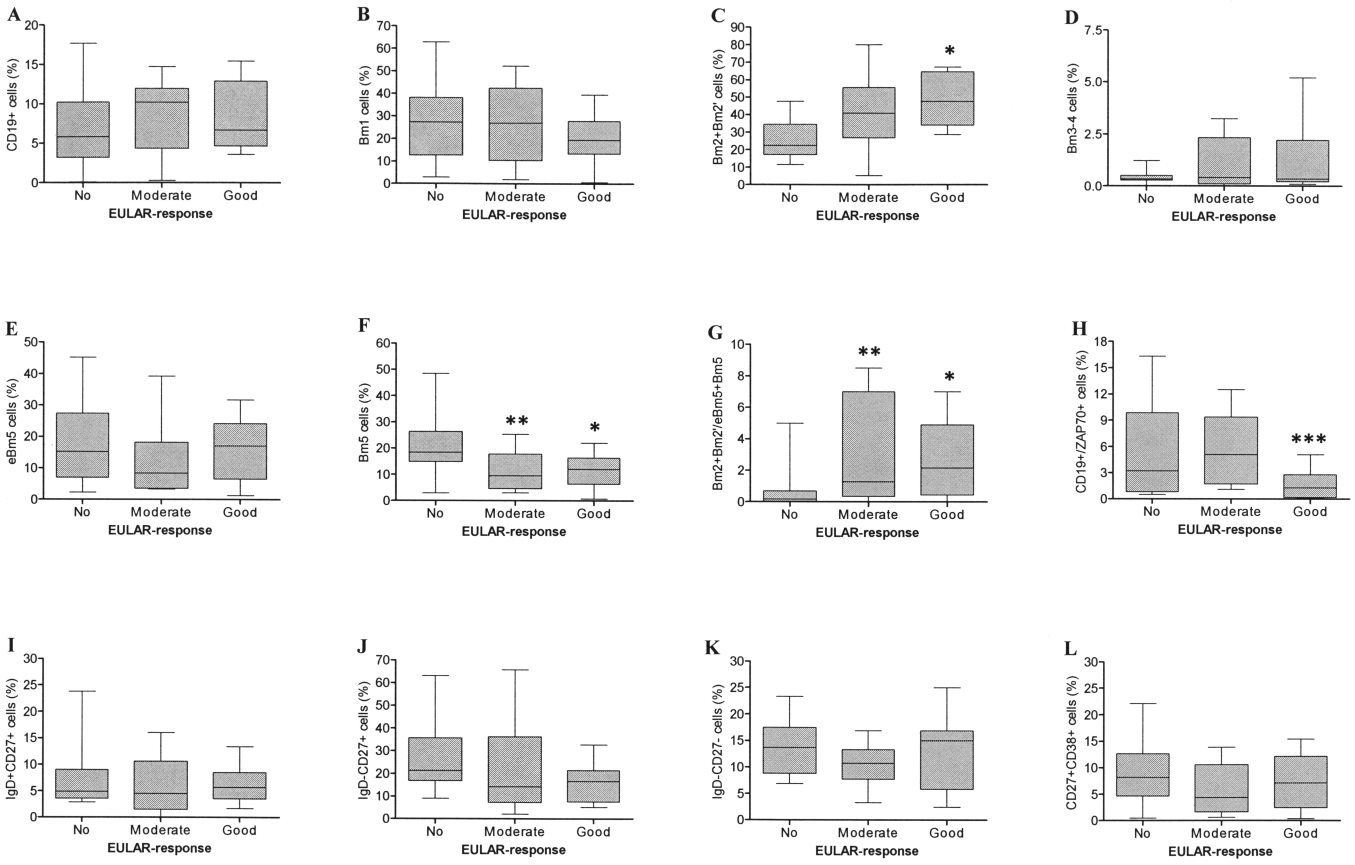

Figure 2. Baseline distribution of B cell subsets in patients with rheumatoid arthritis (RA) according to European League Against Rheumatism (EULAR) response at the 6-month followup. B cell analysis was by the flow cytometry method. A region was defined around the CD45-positive cells and total B cells were identified based on expression of the cell-surface marker CD19. B cell subpopulations were classified according to IgD and CD38 expression (Bm1-Bm5 classification) or the IgD and CD27 expression[16,25]. *p < 0.05: good responders versus nonresponders; **p < 0.05: moderate responders versus nonresponders; ***p < 0.05: good versus moderate responders.

0.03), Bm2+Bm2'/eBm5+Bm5 ratio > 1.08 (p = 0.06), and CD19+/ZAP-70+ < 5.0% (p = 0.10).

The best independent predictor of a good EULAR response 6 months after BCDT was CD19+/ZAP70+ < 5.0% (OR 1.12, 95% CI 1.04–125.0) in the logistic regression analysis.

B cell subset distribution after the BCDT and clinical response in patients with RA. B cell subset distributions in

patients with RA with detectable CD19+ cells after the 6- and 12-month followups are shown in Figure 3.

Considering only the patients with detectable B cells, the percentage of CD19+ cells was lower at the 6-month followup (1.5% ± 1.2%) and at the 12-month followup (2.6% ± 1.9%) after the BCDT compared to baseline (7.9% ± 3.8%; $p < 0.001$ vs the 6-mo and $p = 0.002$ vs the 12-mo followup, respectively). Conversely, a significantly higher percentage of Bm2' at the 6-month followup (24.2% ± 17.7%) and the 12-month followup (21.7% ± 18.6%) was observed compared to baseline (1.6% ± 1.7%; $p < 0.001$ vs T6 and $p = 0.004$ vs T12). Moreover, the percentage of the Bm3-4 subset was higher at the 6-month followup compared to baseline (0.89% ± 1.1% at baseline vs 6.1% ± 11.3% at T6; $p = 0.003$). According to this finding, a significantly lower percentage of memory B cells, both early and late, was observed after the BCDT compared to baseline values [eBm5: T0 = 15.6% ± 11.7%, T6 = 9.4% ± 13.5%, T12 = 3.5% ± 1.4% ($p = 0.03$ T6 vs T0, $p = 0.01$ T12 vs T0, $p = 0.02$ T12 vs T6); Bm5: T0 = 13.5% ± 8.4%, T6 = 9.1% ± 7.4%, T12 = 4.0% ± 2.8% ($p = 0.04$ T12 vs T0, $p = 0.03$ T12 vs T6)]. Considering only the memory B cell compartment, we found a lower percentage over time, not only of post-switched-memory B cells [T0 = 19.0% ± 10.7% and T12 = 5.3% ± 3.0% ($p = 0.01$)], but also of pre-switched memory B cells [T0 = 6.4% ± 5.5%, T6 = 2.1% ± 2.2%, T12 = 1.9% ± 2.5% ($p = 0.001$ T6 vs T0, $p = 0.003$ T12 vs T0)]. We found the same results for both CD19+/IgD-CD27– cells [T0 = 12.4% ± 5.0%, T6 = 6.2% ± 5.8%, T12 = 3.9% ± 3.5% ($p = 0.003$ T6 vs T0, $p = 0.01$ T12 vs T0, $p = 0.05$ T6 vs T12)] and plasmablasts [CD19+/CD27+CD38+: T0 = 6.8% ± 4.8%, T6 = 4.3% ± 3.6%, T12 = 1.8% ± 1.1% ($p = 0.01$ T12 vs T0, $p = 0.02$ T6 vs T12)]. Finally, there was a significant increase in the percentage of CD19+/ZAP-70-positive B cells over time after the BCTD (Figure 3 and Appendix 1).

DISCUSSION

Biological agents allow us to target different molecular pathways involved in inflammation and autoimmunity, but these drugs are associated with risks and side effects and their costs are high. Therefore, the choice of which drugs to use as first-line treatment or as rescue therapy after the failure of a first biologic would be assisted by biomarkers to predict the response to therapy and to identify which patient could be the best for each single approach[27,28].

Since the introduction of BCDT, a large body of research

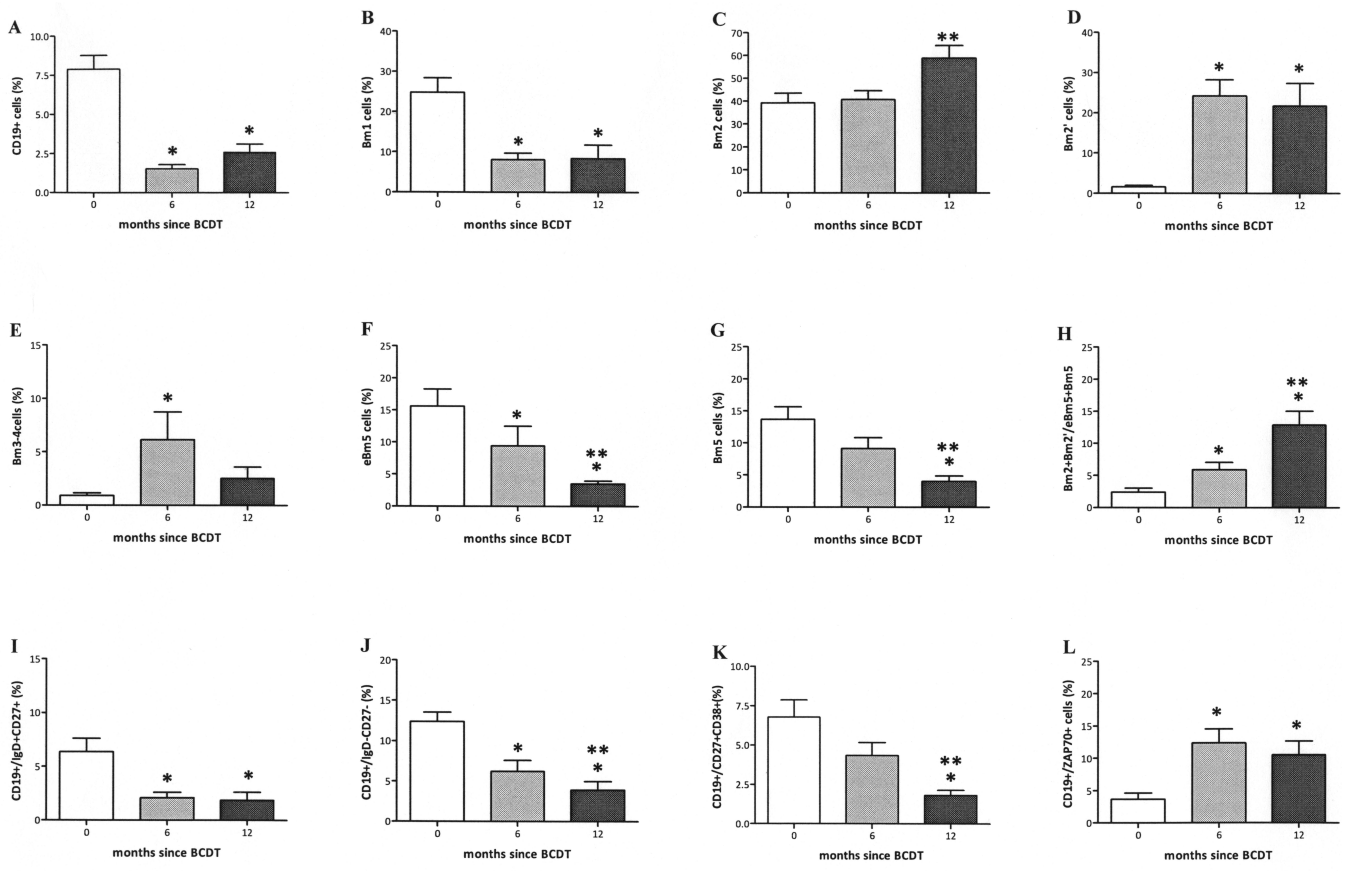

Figure 3. Distribution of B cell subsets according to IgD and CD38 expression (Bm1-Bm5 classification) or IgD and CD27 expression[16,25] in patients with rheumatoid arthritis at baseline and after 6- and 12-month followup since B cell depletion therapy (BCDT). *$p < 0.05$: T6 versus T0 and T12 versus T0; **$p < 0.05$: T12 versus T6.

has been carried out to identify mechanisms of depletion and regeneration of the B cell pool. The reconstitution of the peripheral blood B cell pool follows a typical pattern characterized by the appearance of immature (antigen-inexperienced) CD38++IgD+ B cells. Therefore, a continuous increase of naive B cells and later a deep and long-lasting reduction of memory B cells occur[29,30]. The 2-phase process appears to be present in the majority of patients and it is thought to be fundamental in restoring immune system and tolerance. While the association between B cell subsets after BCDT and response to therapy has been widely documented in RA, no clear data are available regarding the predictive value of studying the baseline B cell compartments. Recently, Sellam, *et al* showed an association between a low frequency of un-switched and switched memory B cells and a better clinical response to a single course of RTX in a large RA cohort[31]. Our data partially confirm these results, since we found that RA patients with a good EULAR response showed a higher baseline percentage of naive-activated B cells (Bm2) compared to nonresponders. According to multivariate analysis, however, in our RA patient cohort, a low percentage of peripheral blood ZAP-70+ B cells (< 5.0%) stood out as the only predictive factor for response to the BCDT at the 6-month followup among all B cell subsets at baseline. The percentage of peripheral blood ZAP-70+ B cells was tightly correlated at baseline to the inflammatory milieu, i.e., plasma IL-6 levels. The data confirmed our previous findings that ZAP70+ B cells were linked to the inflammatory and autoimmune phenotype. We showed CD27+/IgD–/ZAP70+ memory B cells were found to accumulate preferentially in the joints of patients with RA and were associated with a dynamic maturation of B cells according to disease activity and duration[21,22].

The depletion of memory B cells in peripheral blood and bone marrow has been demonstrated to precede clinical response in RA patients treated with RTX[30]. Moreover, the reduction of resident CD27+ B cells in bone marrow is a feature of the longterm effect of RTX and it does not appear to be able to disrupt the regeneration of autoreactive B cell clones[32,33].

Our results are in agreement with the observations previously reported, since they confirm how RTX determines a progressive decrease of memory subsets in the longterm followup. We observed, instead, B cells lacking the expression of CD27 and IgD, i.e., double-negative memory B cells, are affected by BCDT, showing lower levels after the 6- and 12-month followups starting from baseline treatment. The CD19+/CD27–IgD– cells were first described in patients with systemic lupus erythematosus, and were characterized not only by an activating phenotype, but also by an association with a worse clinical outcome and an occurrence of disease flares[33]. In our RA cohort, the CD19+/CD27–IgD– cells were not helpful in distinguishing patients according to therapy response.

Plasmablasts significantly decreased after the BCDT in our RA cohort and did not show different baseline levels in responders compared to nonresponders. In responder patients, plasmablasts were recently found to be associated with a better response to RTX[12].

To define the features of peripheral blood B cell pool regeneration after BCDT, we studied intracellular ZAP-70 expression. We found that an increasing proportion of B cells could be identified as CD19+/ZAP-70+ cells during repopulation. Recent data demonstrated that B cells carrying ZAP-70 could express a more activated-aggressive phenotype in CLL[34,35].

Our study focused on the features of circulating B cells at baseline and after repopulation. It supplies information about the presence of CD19+/ZAP-70+ cells in RA. The presence of the CD19+/ZAP-70+ cell subset has emerged as a baseline predictor of short-term efficacy of BCDT. In particular, patients with RA exhibiting a poor response to BCDT after 6 months showed significantly higher pretreatment percentages of CD19+/ZAP-70+ cells compared to good responders. It is crucial to identify whether the expression of ZAP-70 protein is either constitutive or induced and inducible, and therefore, whether this particular subset of B cells could represent a possible mechanism of resistance to BCDT.

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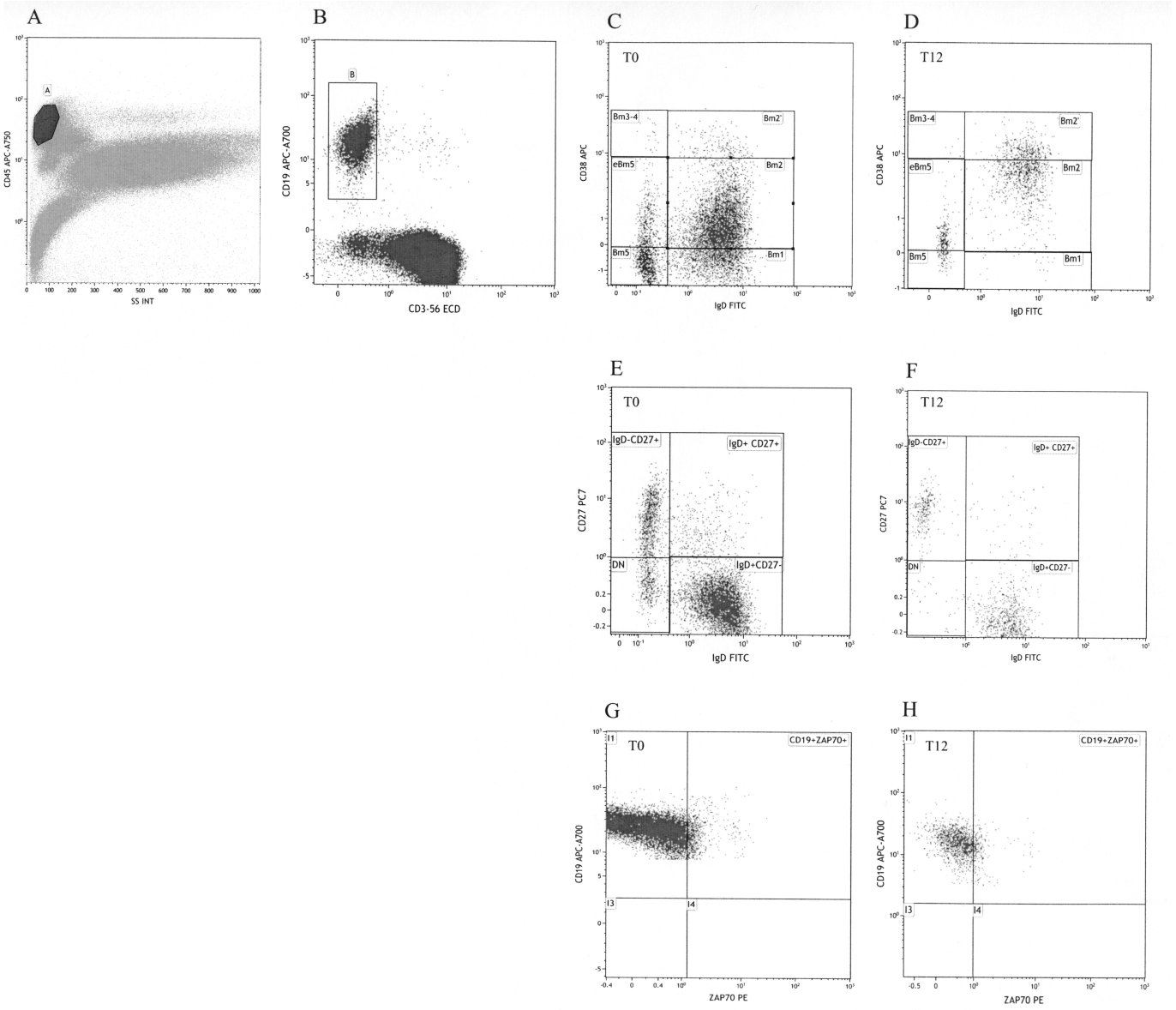
REFERENCES

1. Quinn MA, Emery P. Window of opportunity in early rheumatoid arthritis: Possibility of altering the disease process with early intervention. Clin Exp Rheumatol 2003;21:S154-7.
2. van de Sande MG, de Hair MJ, van der Leij C, Klarenbeek PL, Bos WH, Smith MD, et al. Different stages of rheumatoid arthritis: Features of the synovium in the preclinical phase. Ann Rheum Dis 2011;70:772-7.
3. Hazes JM, Luime JJ. The epidemiology of early inflammatory arthritis. Nat Rev Rheumatol 2011;7:381-90.
4. Martin F, Chan AC. B cell immunobiology in disease: Evolving concepts from the clinic. Annu Rev Immunol 2006;24:467-96.
5. Edwards JC, Leandro MJ, Cambridge G. B lymphocyte depletion in rheumatoid arthritis: Targeting of CD20. Curr Dir Autoimmun 2005;8:175-92.
6. Hoyer BF, Manz RA, Radbruch A, Hiepe F. Long-lived plasma cells and their contribution to autoimmunity. Ann NY Acad Sci 2005;1050:124-33.
7. De Vita S, Zaja F, Sacco S, De Candia A, Fanin R, Ferraccioli G. Efficacy of selective B cell blockade in the treatment of rheumatoid arthritis: Evidence for a pathogenetic role of B cells. Arthritis Rheum 2002;46:2029-33.
8. Chynes RA, Towers TL, Presta LG. Inhibitory Fc receptors modulate in-vivo cytotoxicity against tumor targets. Nat Med 2006;6:443-6.
9. Thurlings RM, Vos K, Wijbrandts CA, Zwinderman AH, Gerlag DM, Tak PP. Synovial tissue response to rituximab: Mechanism of action and identification of biomarkers of response. Ann Rheum Dis 2008;67:917-25.



Appendix 1. Dot plots show B cell subsets and ZAP-70 protein expression in peripheral blood from a representative patient with rheumatoid arthritis (RA) before (T0) and after B cell depletion therapy (T12). A region was defined around the CD45-positive cells (gate A, plot A) and total B cells were identified based on expression of the cell-surface marker CD19 (gate B, plot B). B cell subsets were classified according to IgD and CD38 expression (Bm1-Bm5 classification; plot C at baseline and plot D at 12-month followup period) or the IgD and CD27 expression (plot E at baseline and plot F at 12-mo followup period)[16,25]. Dot plots G (baseline) and H (at 12-mo followup period) illustrate ZAP-70 protein expression in B cells from a representative patient with RA. ZAP-70 was evaluated using T and natural killer cells as internal reference[21,26].

10. Vos K, Thurlings RM, Wijbrandts CA, van Schaardenburg DJ, Gerlag DM, Tak PP. Early effects of rituximab on the synovial cell infiltrate in patients with rheumatoid arthritis. Arthritis Rheum 2007;56:772–8.
11. Chatzidionysiou K, Lie E, Nasonov E, Lukina G, Hetland ML, Tarp U, et al. Highest clinical effectiveness of rituximab in autoantibody-positive patients with rheumatoid arthritis and in those for whom no more than one previous TNF antagonist has failed: Pooled data from 10 European registries. Ann Rheum Dis 2011;70:1575-80.
12. Vital EM, Rawstron AC, Dass S, Henshaw K, Madden J, Emery P, et al. Reduced-dose rituximab in rheumatoid arthritis: Efficacy depends on degree of B cell depletion. Arthritis Rheum 2011;63:603-8.
13. Edwards JC, Szczepanski L, Szechinski J, Filipowicz-Sosnowska A, Emery P, Close DR, et al. Efficacy of B-cell-targeted therapy with rituximab in patients with rheumatoid arthritis. N Engl J Med 2004;350:2572–81.
14. Emery P, Fleischmann R, Filipowicz-Sosnowska A, Schechtman J, Szczepanski L, Kavanaugh A, et al. The efficacy and safety of rituximab in patients with active rheumatoid arthritis despite methotrexate treatment: Results of a phase IIB randomized, double-blind, placebo-controlled, dose-ranging trial. Arthritis Rheum 2006;54:1390-400.
15. Cohen SB, Emery P, Greenwald MW, Dougados M, Furie RA, Genovese MC, at al. Rituximab for rheumatoid arthritis refractory

Appendix 2. B cell subset distribution in RA patients previously treated only with DMARD and anti-TNF before B cell depletion therapy compared to healthy controls. Values are mean ± SD.

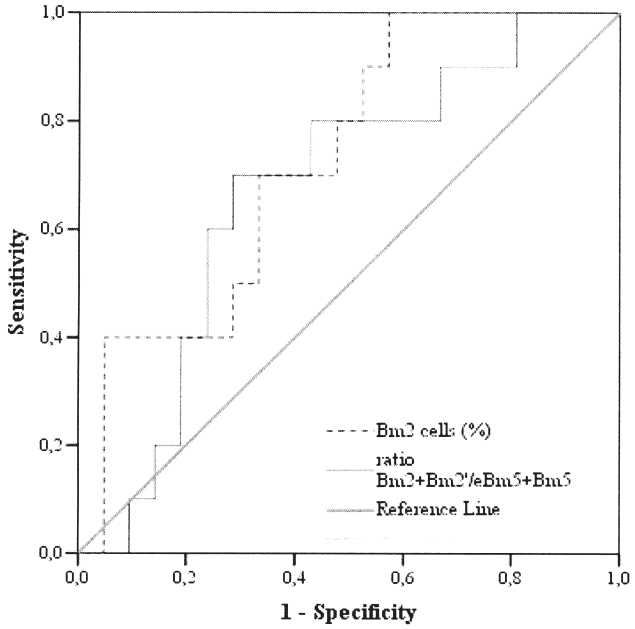
Subset	Controls, N = 30	Anti-TNF-α Group, N = 21	DMARD Group, N = 10	p[a]	p[b]	p[c]
White blood cells	6047.3 ± 1288.7	8147.0 ± 3001.2	6531.1 ± 2015.4	0.01	0.70	0.15
Lymphocytes, %	33.2 ± 8.1	31.3 ± 12.8	37.4 ± 14.4	0.41	0.50	0.23
Lymphocytes/mm^3	1932.9 ± 384.5	2397.4 ± 1100.7	2379.4 ± 1025.6	0.18	0.27	0.98
CD19+ B cells, %	9.6 ± 2.9	7.6 ± 4.5	8.0 ± 5.0	0.06	0.35	0.80
CD19+ B cells, n	181.1 ± 71.2	175.6 ± 148.5	184.9 ± 109.9	0.41	0.84	0.67
Bm1, %	23.3 ± 10.4	26.2 ± 16.3	22.0 ± 14.9	0.37	0.30	0.44
Bm2, %	41.2 ± 11.9	38.6 ± 13.8	34.4 ± 25.2	0.38	0.27	0.39
Bm2′, %	1.7 ± 1.1	1.3 ± 1.6	1.9 ± 2.7	0.05	0.34	0.66
Bm2+Bm2′, %	43.0 ± 12.5	39.9 ± 14.5	36.4 ± 25.3	0.33	0.34	0.47
Bm3–4, %	0.7 ± 0.4	0.9 ± 1.4	0.8 ± 0.9	0.04	0.90	0.15
eBm5, %	16.0 ± 7.1	16.8 ± 11.8	11.9 ± 8.9	0.97	0.08	0.37
Bm5, %	15.1 ± 6.5	14.1 ± 8.4	23.1 ± 22.3	0.57	0.64	0.39
eBm5+Bm5, %	31.1 ± 12.1	30.1 ± 13.6	33.7 ± 26.1	0.80	0.55	0.79
Ratio Bm2+Bm2/eBm5+Bm5	1.6 ± 0.9	1.8 ± 2.5	2.6 ± 2.8	0.07	0.88	0.40
CD19+/IgD+CD27–	60.8 ± 11.8	57.4 ± 13.4	56.9 ± 25.2	0.25	1.0	0.65
CD19+/IgD+CD27+	6.7 ± 3.4	8.2 ± 5.9	3.7 ± 2.5	0.79	0.02	0.02
CD19+/IgD–CD27+	16.3 ± 7.0	12.1 ± 15.2	14.6 ± 22.1	0.35	0.35	0.83
CD19+/IgD–CD27–	16.2 ± 9.1	12.9 ± 5.5	11.4 ± 5.0	0.23	0.28	0.61
CD19+/CD27+CD38+	8.0 ± 3.5	7.2 ± 4.9	7.6 ± 6.9	0.46	0.25	0.97
CD19+/ZAP-70+	2.2 ± 1.4	4.8 ± 4.9	3.8 ± 3.9	0.46	0.44	0.54
CD19+/CD5+, %	13.8 ± 8.5	11.0 ± 6.1	4.2 ± 2.1	0.63	0.003	0.03

TNF: tumor necrosis factor; DMARD: disease-modifying antirheumatic drugs; RA: rheumatoid arthritis. [a] previous anti-TNF-α RA patients vs controls; [b] previous DMARD RA patients vs controls; [c] previous anti-TNF-α vs previous DMARD RA patients.

Appendix 3. B cell subset distribution before B cell depletion therapy according to the EULAR response at 6-month followup in RA patients. Values are mean ± SD.

Subset	Controls, N = 30	RA Patients DAS > 2.4, N = 21	RA Patients DAS ≤ 2.4, N = 10	RA Patients DAS > 3.7	RA Patients DAS ≤ 3.7	p[a]	p[b]	p[c]	p[d]	p[e]	p[f]
White blood cells	6047.3 ± 1288.7	7919.5 ± 3181.9	7125.0 ± 1928.2	8291.8 ± 2966.6	7250.5 ±	0.65	0.19	0.04	0.18	0.16	0.18
Lymphocytes, %	33.2 ± 8.1	32.0 ± 11.8	35.5 ± 16.3	32.5 ± 13.3	33.6 ± 13.8	0.68	0.82	0.66	0.98	0.70	0.83
Lymphocytes/mm^3	1932.9 ± 384.5	2270.8 ± 742.4	2621.8 ± 1516.8	2500.7 ± 845.8	2325.3 ±	0.65	0.42	0.10	0.53	0.02	0.65
CD19+ B cells, %	9.6 ± 2.9	7.4 ± 4.8	8.5 ± 4.4	6.6 ± 4.9	8.4 ± 4.4	0.52	0.27	0.06	0.39	0.03	0.33
Bm1, %	23.3 ± 10.4	27.1 ± 17.5	20.2 ± 10.6	28.5 ± 18.6	22.9 ± 14.1	0.37	0.43	0.57	0.63	0.49	0.84
Bm2, %	41.2 ± 11.9	32.9 ± 18.3	46.5 ± 14.2	25.7 ± 12.7	43.6 ± 17.5	0.04	0.01	0.04	0.38	0.003	0.70
Bm2′, %	1.7 ± 1.1	1.0 ± 1.2	2.6 ± 2.9	0.9 ± 1.0	1.9 ± 2.3	0.18	0.47	0.01	0.99	0.02	0.31
Bm2+Bm2′, %	43.0 ± 12.5	33.9 ± 18.2	49.0 ± 14.2	26.6 ± 12.5	45.4 ± 17.7	0.02	0.01	0.03	0.27	0.002	0.61
Bm3–4, %	0.7 ± 0.4	0.7 ± 0.9	1.2 ± 1.7	0.4 ± 0.3	1.1 ± 1.5	0.74	0.63	0.12	0.49	0.07	0.40
eBm5, %	16.0 ± 7.1	14.9 ± 11.9	16.0 ± 9.6	17.6 ± 12.3	13.9 ± 10.4	0.55	0.36	0.24	0.94	0.92	0.22
Bm5, %	15.1 ± 6.5	19.5 ± 16.9	11.7 ± 6.2	21.3 ± 11.9	14.6 ± 15.7	0.18	0.03	0.60	0.20	0.08	0.14
eBm5+Bm5, %	31.1 ± 12.1	33.8 ± 20.1	25.9 ± 12.5	37.6 ± 18.5	27.8 ± 17.5	0.31	0.14				
Ratio Bm2+Bm2/eBm5+Bm5	1.6 ± 0.9	1.8 ± 2.7	2.7 ± 2.4	0.7 ± 1.5	2.8 ± 2.8	0.13	0.01	0.02	0.32	< 0.001	0.54
CD19+/IgD+CD27–	60.8 ± 11.8	53.5 ± 19.2	64.5 ± 13.1	51.0 ± 17.4	61.3 ± 17.5	0.14	0.51	0.13	0.56	0.49	0.90
CD19+/IgD+CD27+	6.7 ± 3.4	7.0 ± 6.2	6.2 ± 3.4	7.9 ± 7.1	6.0 ± 4.3	0.75	0.61	0.41	0.54	0.49	0.43
CD19+/IgD–CD27+	16.3 ± 7.0	23.6 ± 17.0	16.2 ± 8.2	25.7 ± 14.9	18.8 ± 14.9	0.25	0.09	0.16	0.91	0.03	0.90
CD19+/IgD–CD27–	16.2 ± 9.1	12.0 ± 4.6	13.3 ± 6.7	13.3 ± 5.0	11.9 ± 5.5	0.38	0.62	0.10	0.71	0.95	0.14
CD19+/CD27+CD38+	8.0 ± 3.5	7.3 ± 5.8	7.3 ± 5.2	8.8 ± 6.4	6.5 ± 4.9	0.95	0.34	0.28	0.53	0.08	0.12
CD19+/ZAP-70+	2.2 ± 1.4	5.6 ± 4.9	1.8 ± 1.7	5.6 ± 5.6	3.7 ± 3.6	0.03	0.47	0.08	0.32	0.31	0.57
CD19+/CD5+, %	13.8 ± 8.5	9.9 ± 6.7	7.3 ± 4.5	8.9 ± 5.7	9.3 ± 6.6	0.66	0.89	0.24	0.11	0.22	0.17

[a] Good vs poor responders at 6 months of therapy; [b] moderate vs nonresponders at 6 months of therapy; [c] poor responders vs healthy controls; [d] good responders vs healthy controls; [e] nonresponders vs healthy controls; [f] moderate responders vs healthy controls. EULAR: European League Against Rheumatism; RA: rheumatoid arthritis; DAS: Disease Activity Score.



Appendix 4. Receiver-operating curve (ROC) analyses were used to evaluate the cutoff value of biomarkers (Bm2 and ratio of Bm2+Bm2'/eBm5 +Bm5) as predictors of a good EULAR response. Area under the ROC curve for the Bm2 biomarker was 0.729 ± 0.092 (p = 0.04) and for the Bm2+Bm2'/eBm5+Bm5 ratio was 0.671 ± 0.102 (p = 0.13).

to anti-tumor necrosis factor therapy: Results of a multicenter, randomized, double-blind, placebo-controlled, phase III trial evaluating primary efficacy and safety at twenty-four weeks. Arthritis Rheum 2006;54:2793-806.

16. Anolik JH, Looney RJ, Lund FE, Randall TD, Sanz I. Insights into the heterogeneity of human B cells: Diverse functions, roles in autoimmunity, and use as therapeutic targets. Immunol Res 2009,45:144-58.
17. Silverman GJ. Therapeutic B cell depletion and regeneration in rheumatoid arthritis: Emerging patterns and paradigms. Arthritis Rheum 2006;54:2356-67.
18. Roll P, Palanichamy A, Kneitz C, Dorner T, Tony HP. Regeneration of B cell subsets after transient B cell depletion using anti-CD20 antibodies in rheumatoid arthritis. Arthritis Rheum 2006; 54:2377-86.
19. Elder ME, Lin D, Clever J, Chan AC, Hope TJ, Weiss A, et al. Human severe combined immunodeficiency due to a defect in ZAP-70, a T cell tyrosine kinase. Science 1994;264:1596-9.
20. Gobessi S, Laurenti L, Longo PG, Sica S, Leone G, Efremov DG. ZAP-70 enhances B-cell-receptor signaling despite absent or inefficient tyrosine kinase activation in chronic lymphocytic leukemia and lymphoma B cells. Blood 2007;109:2032-9.
21. Tolusso B, De Santis M, Bosello S, Gremese E, Gobessi S, Cuoghi I, et al. Synovial B cells of rheumatoid arthritis express ZAP-70 which increases the survival and correlates with the inflammatory and autoimmune phenotype. Clin Immunol 2009;131:98-108.
22. Michelutti A, Gremese E, Morassi F, Petricca L, Arena V, Tolusso B, et al. B cell subsets in the joint compartments of seropositive and seronegative RA and non-RA arthritides express memory markers and ZAP-70 and characterize the aggregate pattern irrespectively of the autoantibody status. Mol Med 2011;17:901-9.
23. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315-24.
24. van Gestel AM, Prevoo ML, van 't Hof MA, van Rijswijk MH, van de Putte LB, van Riel PL. Development and validation of the European League Against Rheumatism response criteria for rheumatoid arthritis. Comparison with the preliminary American College of Rheumatology and the World Health Organization/International League Against Rheumatism Criteria. Arthritis Rheum 1996;39:34-40.
25. Youinou P. B cells conduct the lymphocyte orchestra. J Autoimmunity 2007;28:143-51.
26. Crespo M, Bosch F, Villamor N, Bellosillo B, Colomer D, Rozman M, et al. ZAP-70 expression as a surrogate for immunoglobulin-variable-region mutations in chronic lymphocytic leukemia. N Engl J Med 2003;348:1764-75.
27. Isaacs JD, Ferraccioli G. The need for personalised medicine for rheumatoid arthritis. Ann Rheum Dis 2011;70:4-7.
28. Buch MH, Rubbert-Roth A, Ferraccioli G. To switch or not to switch after a poor response to a TNF-α blocker? It is not only a matter of ACR20 OR ACR50. Autoimmun Rev 2012;11:558-62.
29. Roll P, Dörner T, Tony HP. Anti-CD20 therapy in patients with rheumatoid arthritis: Predictors of response and B cell subset regeneration after repeated treatment. Arthritis Rheum 2008;58:1566-75.
30. Nakou M, Katsikas G, Sidiropoulos P, Bertsias G, Papadimitraki E, Raptopoulou A, et al. Rituximab therapy reduces activated B cells in both the peripheral blood and bone marrow of patients with rheumatoid arthritis: Depletion of memory B cells correlates with clinical response. Arthritis Res Ther 2009;11:R131.
31. Sellam J, Rouanet S, Hendel-Chavez H, Abbed K, Sibilia J, Tebib J, et al. Blood memory B cells are disturbed and predict the response to rituximab in patients with rheumatoid arthritis. Arthritis Rheum 2011;63:3692-701.
32. Rehnberg M, Amu S, Tarkowski A, Bokarewa MI, Brisslert M. Short- and long-term effects of anti-CD20 treatment on B cell ontogeny in bone marrow of patients with rheumatoid arthritis. Arthritis Res Ther 2009;11:R123.
33. Anolik JH, Barnard J, Cappione A, Pugh-Bernard AE, Felgar RE, Looney RJ, et al. Rituximab improves peripheral B cell abnormalities in human systemic lupus erythematosus. Arthritis Rheum 2004;50:3580-90.
34. Calpe E, Codony C, Baptista MJ, Abrisqueta P, Carpio C, Purroy N, et al. ZAP-70 enhances migration of malignant B lymphocytes toward CCL21 by inducing CCR7 expression via IgM-ERK1/2 activation. Blood 2011;118:4401-10.
35. Chen L, Huynh L, Apgar J, Tang L, Rassenti L, Weiss A, et al. ZAP-70 enhances IgM signaling independent of its kinase activity in chronic lymphocytic leukemia. Blood 2008;111:2685-92.