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# The TTTT B Lymphocyte Stimulator Promoter Haplotype Is Associated With Good Response to Rituximab Therapy in Seropositive Rheumatoid Arthritis Resistant to Tumor Necrosis Factor Blockers

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***Objective.*** **To investigate the polymorphisms in the promoter region of the B lymphocyte stimulator (BLyS) gene as markers of response to rituximab (RTX) in rheumatoid arthritis (RA).**

***Methods.*** **The study was first conducted in 152 Italian RA patients and then replicated in an additional 117 RA patients (73 Italian, 44 British). The European League Against Rheumatism response criteria were used to evaluate the response rate at months 4 and 6 after the first cycle of RTX, by means of the Disease Activity Score in 28 joints using the erythrocyte sedimentation rate; patients were classified according to the best response shown between months 4 and 6. BLyS promoter polymorphisms were analyzed by polymerase chain reaction followed by the analysis of the restriction fragments, BLyS promoter haplotypes were analyzed using the expectation-maximization algorithm, and BLyS serum levels were analyzed using enzyme-linked immunosorbent assay. Odds ratios (ORs) were calculated with 95% confidence intervals (95% CIs).**

***Results.*** **The TTTT BLyS promoter haplotype appeared to be significantly associated with response to RTX only in the subset of seropositive patients (those positive for rheumatoid factor and/or anti–cyclic citrullinated peptide). The replication study confirmed that this association was limited to seropositive RA patients in whom treatment with anti–tumor necrosis factor**

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**(anti-TNF) agents had previously failed. In the whole series of seropositive patients in whom anti-TNF agents had previously failed, patients carrying the TTTT BLyS promoter haplotype were more prevalent in good responders (18 of 43 [41.9%]) than in moderate responders (20 of 83 [24.1%]) or in nonresponders (1 of 21 [4.8%]) (for good responders versus nonresponders, OR 14.4 [95% CI 1.77–117.39], $P = 0.0028$). Furthermore, multivariate analysis selected the TTTT BLyS promoter haplotype as an independent marker of good response to RTX (for good responders versus nonresponders, OR 16.2 [95% CI 1.7–152.5], $P = 0.01$; for good responders versus moderate responders and nonresponders combined, OR 3.1 [95% CI 1.2–7.8], $P = 0.02$). The relationship between BLyS polymorphisms and BLyS serum levels remained unclear.**

***Conclusion*. BLyS promoter genotyping may be suitable for identifying seropositive RA patients who may have a good response to RTX after anti-TNF agents have failed.**

Rituximab (RTX), a chimeric monoclonal antibody directed at the CD20 membrane protein present on B cells (1), is an effective treatment for patients with rheumatoid arthritis (RA), especially those positive for rheumatoid factor (RF) and/or anti–cyclic citrullinated peptide (anti-CCP) antibodies (2–5). Several studies demonstrated its efficacy for the treatment of RA, including RA unresponsive to anti–tumor necrosis factor (anti-TNF) therapy, underlying the important role of B cells in this disease (6–10). However, among RA patients who respond to RTX, some have relapse of their disease while others show a prolonged response for a very long time, independently of the reappearance of B cells in the peripheral blood (usually after 6–8 months) (9). One possible explanation may reside in the differential effects of RTX in depleting B cells and blocking plasma cell generation at the synovial and bone marrow levels, as recently demonstrated (11–13). However, the degree and duration of response to RTX may also depend on individual genetic predisposition.

B lymphocyte stimulator (BLyS), also called BAFF, is a key cytokine in B cell survival and proliferation (14), and elevated serum and tissue levels were described in several autoimmune diseases, including RA (15,16). The link between BLyS serum levels and BLyS genetics has been investigated more rarely in rheumatic diseases, mainly by analyzing the polymorphism –871C/T in the BLyS promoter region. The –871T allele was associated with higher BLyS serum levels, but reported data are limited and patient selection not well specified (17–19). Of note, BLyS levels further increase soon after RTX infusion (20,21), possibly contributing to the reappearance of B cells and affecting the duration and degree of RTX response. Thus, the regulation of BLyS expression may be linked to the efficacy of RTX.

In this work we extended an initial study of the –871C/T BLyS polymorphism (22) to all the common polymorphisms located in the 5′ regulatory region of the BLyS gene, which are in strong linkage disequilibrium and form 4 major haplotypes (23). The study was first conducted in a large retrospective cohort of Italian RA patients treated with RTX. Response rates were analyzed both at month 4 and at month 6 after RTX treatment, and the best response shown between months 4 and 6 was chosen to identify 3 response subgroups: good responders, moderate responders, and nonresponders. In addition, we considered 3 established items related to response to RTX (RF positivity, anti-CCP positivity, and previous anti-TNF$\alpha$ therapy [2]) to better dissect the possible predictive role of the genetic data in different subgroups of RA patients. Finally, a replication study was conducted in an additional 117 unselected RA patients. The possible correlation between BLyS genetics and BLyS serum levels was also investigated.

## PATIENTS AND METHODS

**Study populations.** The study was first conducted in a retrospective cohort of 152 unselected RA patients who had been referred to 6 rheumatology centers in Italy. The replication series comprised 117 additional, unselected RA patients, 73 of whom were referred to 7 different Italian rheumatology centers, and 44 of whom were British patients followed up at the University of Leeds. All RA patients were diagnosed according to the 1987 American College of Rheumatology revised classification criteria (24). All patients gave their informed consent to participation in the study, in accordance with the Declaration of Helsinki, and the investigation was approved by the local study review boards.

Demographic and clinical features of the study patients are illustrated in Table 1. Patients included in these 2 series represent the large majority of RA patients treated with RTX in those centers, and all of them were followed up for at least 6 months after RTX treatment. All patients were taking $<10$ mg/day of prednisone or equivalent, and no increase in the corticosteroid dosage was allowed after RTX therapy was begun. All patients were treated with intravenous infusions of RTX (500 mg at weeks 0, 1, and 2 or 1,000 mg at weeks 0 and 2) either as monotherapy (4.1%) or in combination with either methotrexate (MTX) or other disease-modifying antirheumatic drugs (DMARDs), such as leflunomide, cyclosporin A, or hydroxychloroquine (95.9%). In the majority of patients, treatment with 1 or more anti-TNF agents had failed due to primary or secondary inefficacy. In the remaining patients, the disease was unresponsive to at least 6 months of treatment with MTX alone or in combination with other DMARDs with RTX

**Table 1.** Baseline characteristics of the study patients*

| | First series of patients (n = 152) | Replication series of patients (n = 117) |
|---|---|---|
| Demographics | | |
| Female | 127 (83.6) | 102 (87.2) |
| Age, mean ± SD (range) years | 61 ± 13 (23–84) | 57 ± 12 (29–84) |
| Disease status | | |
| RF positive | 123 (80.9)† | 81 (69.2) |
| Anti-CCP positive | 120 (78.9)‡ | 82 (70.1) |
| RF positive and/or anti-CCP positive | 132 (86.8) | 97 (82.9) |
| DAS28-ESR, mean ± SD (range) | 5.91 ± 1.11 (3.10–8.38) | 5.85 ± 1.31 (2.68–8.52) |
| Disease duration, mean ± SD (range) years | 12.3 ± 9.5 (1–44) | 15.5 ± 11.4 (1–56) |
| Medications | | |
| Concomitant DMARDs | 149 (98) | 109 (93.4) |
| Concomitant MTX | 127 (83.6) | 96 (82.1) |
| Anti-TNF naive | 40 (26.3)§ | 50 (42.7) |
| Number of biologic agents before RTX, mean ± SD (range) | 1.5 ± 0.7 (1–4) | 1.7 ± 0.6 (1–3) |

* Except where indicated otherwise, values are the number (%). RF = rheumatoid factor; anti-CCP = anti–cyclic citrullinated peptide; DAS28-ESR = Disease Activity Score in 28 joints using the erythrocyte sedimentation rate; DMARDs = disease-modifying antirheumatic drugs; MTX = methotrexate; anti-TNF = anti–tumor necrosis factor; RTX = rituximab.
† Odds ratio (OR) 1.81 (95% confidence interval [95% CI] 1.03–3.19), $P = 0.0437$ versus replication series of patients.
‡ OR 1.61 (95% CI 0.92–2.79), $P = 0.12$ versus replication series of patients.
§ OR 2.09 (95% CI 1.25–3.50), $P = 0.0061$ versus replication series of patients.

representing the first biologic therapy. RA patients in whom anti-TNF therapy had failed only because of side effects were excluded from the study.

The European League Against Rheumatism (EULAR) response criteria (25) were used to evaluate response rates at months 4 and 6 after the first cycle of RTX, by means of the Disease Activity Score in 28 joints (26) using the erythrocyte sedimentation rate (DAS28-ESR). Patients were finally classified according to their best response between months 4 and 6 after RTX treatment, since this proved to be more relevant than the single evaluation of response at either month 4 or month 6 to determine whether to proceed with RTX retreatment (9).

**BLyS promoter genotyping.** DNA was extracted from EDTA-treated peripheral blood using an automated method (Maxwell 16; Promega) and dedicated kits (Maxwell 16 Blood DNA purification kit). The single-nucleotide polymorphism (SNP) –871 C>T (rs9514828) was first analyzed following previously reported methods (19). Then, according to the method used by Nossent et al (23), the other 3 SNPs in the haplotype block of the 5′-regulatory region of the BLyS gene were also analyzed: –2841 T>C (rs9514827), –2704 T>C (rs3759467), and –2701 T>A (rs1041569). The analyses of the –2841 T>C (rs9514827), –2704 T>C (rs3759467), and –2701 T>A (rs1041569) polymorphisms were made starting from the same amplicon. All polymerase chain reaction products were digested with 10 units of the appropriate restriction enzyme for at least 2 hours at the temperature recommended by the manufacturer. The restriction fragments were run onto 2% ethidium bromide–stained agarose gels with sodium borate buffer and visualized with a Phosphor Imager (Bio-Rad).

**Enzyme-linked immunosorbent assay (ELISA) for BLyS serum level analysis.** BLyS serum levels were analyzed by ELISA using commercially available kits (Quantikine Human BAFF/BLyS, lower limit of detection 3.38 pg/ml; R&D Systems). Baseline sera were available only from a fraction of RA patients under study (n = 82).

**Statistical analysis.** BLyS haplotypes, defined by the 4 above mentioned SNPs in the promoter region, were analyzed using the freely available hapassoc software, version 1.1 (Simon Fraser University, Burnaby, British Columbia, Canada), which estimates haplotypes using the expectation-maximization algorithm, as indicated by Nossent et al (23). The chi-square test or Fisher's exact test for categorical variables and the Mann-Whitney test for quantitative variables were used, after verifying the assumptions, for univariate analysis. Multivariate analysis was performed by stepwise logistic regression, where age, sex, baseline DAS28-ESR, RF status, anti-CCP status, failure of anti-TNF therapy, and BLyS haplotypes were chosen as covariates to be included in the model to assess the variables predicting EULAR response. All these variables were available in all cases. Odds ratios (ORs) are provided with 95% confidence intervals (95% CIs). Data were analyzed with SPSS software, version 15.1. *P* values less than or equal to 0.05 were considered significant.

## RESULTS

**Response to RTX and genetic studies in the first study population.** The first study population comprised 152 RA patients treated with RTX who presented a high prevalence of seropositivity (86.8% RF positive and/or anti-CCP positive) and a large majority of whom had a history of anti-TNF therapy failure (73.7%). According to the evaluation of the best clinical response between month 4 and month 6, 3 subgroups were identified: good

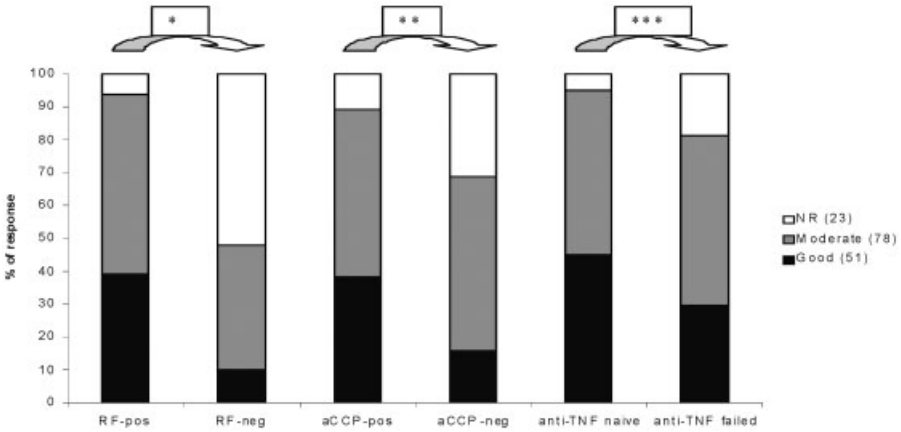


**Figure 1.** Clinical variables associated with response to rituximab in the first study population. Rheumatoid factor (RF)–positive patients showed a higher response rate than did RF-negative patients. For good responders and moderate responders combined versus nonresponders (NR), * = odds ratio (OR) 15.4 (95% confidence interval [95% CI] 5.54–42.80), $P < 0.0001$. Anti–cyclic citrullinated peptide (anti-CCP)–positive patients showed a higher response rate than did anti-CCP–negative patients. For good responders and moderate responders combined versus nonresponders, ** = OR 3.74 (95% CI 1.46–9.61), $P = 0.0098$. Anti–tumor necrosis factor (anti-TNF)–naive patients showed a higher response rate than did patients in whom anti-TNF agents had failed. For good responders and moderate responders combined versus nonresponders, *** = OR 4.39 (95% CI 0.98–19.64), $P = 0.041$.

responders (51/152, 33.6%), moderate responders (78/152, 51.3%), and nonresponders (23/152, 15.1%). After RTX therapy, <1% of CD19+ B cells were depleted between months 1 and 3 in all patients. Neither the baseline CD19+ B cell count nor their relative depletion appeared significantly related to the degree of response (data not shown).

As expected from previous studies (2–4), RF-positive and/or anti-CCP–positive antibody status and naive anti-TNF agent status (no previous therapies with anti-TNF agents before RTX) were significantly associated with a positive (good or moderate versus no response) response to RTX (Figure 1). In particular, the highest percentage of good responders (18/40, 45%) and the lowest percentage of nonresponders (2/40, 5%) were among the anti-TNF–naive patients. No difference in the response rate was noted between patients treated with RTX plus DMARDs and patients treated with RTX alone (data not shown).

The study of the possible influence of BLyS genetic variants on the response to RTX started with the analysis of the prevalence of the –871 C>T BLyS promoter polymorphism. The prevalence of the –871 C>T polymorphism genotypes (CC 23.7%, CT 53.3%, TT 23%) appeared comparable to that previously reported in other RA patient series and in Caucasian controls (17–19,23). As illustrated in Table 2, there was no clear association between the –871 C>T BLyS polymorphism and response to RTX; in particular, the rate of response in homozygous CC and TT patients appeared very similar and differed from that in the heterozygous (CT) patients, where the highest prevalence of nonresponders was found.

The analysis of the other BLyS promoter polymorphisms was then performed. The 5′ regulatory region of the human BLyS gene presents the following 4 polymorphic sites: –2841 T>C (rs9514827), –2704 T>C (rs3759467), –2701 T>A (rs1041569), and the best studied –871 C>T. The 4 SNPs are in strong linkage disequilibrium and form 4 common (frequency >5%) haplotypes: CTAT, TTAC, TTTT, and TCAC. In this first series of RA patients, the cumulative frequencies of the estimated BLyS haplotypes (CTAT 0.283, TTAC 0.270, TTTT 0.168, TCAC 0.161, rare pool 0.119) were

**Table 2.** Prevalence of BLyS genotypes (−871 C>T polymorphism) in the 3 subgroups of response to RTX in the first series of patients (n = 152)*

| Best response to RTX between months 4 and 6 of treatment (n) | BLyS −871 genotypes | | |
|---|---|---|---|
| | CC (n = 36) | CT (n = 81) | TT (n = 35) |
| Good (51) | 11 (30.6) | 28 (34.6) | 12 (34.3) |
| Moderate (78) | 22 (61.1) | 34 (42.0) | 22 (62.9) |
| No response (23) | 3 (8.3) | 19 (23.5) | 1 (2.9) |

* Values are the number (%). BLyS = B lymphocyte stimulator; RTX = rituximab.

**Table 3.** Prevalence of BLyS promoter haplotypes in the 3 subgroups of response to RTX in the first series of patients (n = 152)*

| Best response to RTX between months 4 and 6 of treatment (n) | BLyS promoter haplotypes† | | | | |
|---|---|---|---|---|---|
| | CTAT | TTAC | TTTT | TCAC | Rare pool |
| Good (51) | 0.275 | 0.255 | 0.206 | 0.176 | 0.088 |
| Moderate (78) | 0.269 | 0.282 | 0.167 | 0.147 | 0.135 |
| No response (23) | 0.348 | 0.261 | 0.087 | 0.152 | 0.152 |

* The cumulative prevalence of each haplotype is indicated for each subgroup of response to RTX. The prevalence of the TTTT haplotype was greater in good responders than in nonresponders, although the difference was not significant ($P = 0.097$). See Table 2 for definitions.
† Haplotypes were composed following this order: −2841 T>C (rs9514827), −2704 T>C (rs3759467), −2701 T>A (rs1041569), and −871 C>T (rs9514828).

consistent with those reported by the HapMap project (www.hapmap.org) and observed by Nossent et al (23). The prevalence of BLyS haplotypes did not differ significantly between RF-positive and RF-negative patients or between anti-CCP–positive and anti-CCP–negative patients (data not shown).

When related to the response to RTX (Table 3), the prevalence of the TTTT haplotype gradually decreased, from 0.206 in good responders to 0.167 in moderate responders and to 0.087 in nonresponders. More patients carrying the TTTT haplotype were good responders (19 of 51 [37.3%]) than were moderate responders (22 of 78 [28.2%]) or were nonresponders (3 of 23 [13%]) (for good responders versus nonresponders, OR 3.96 [95% CI 1.04–15.12], $P = 0.05$). No significance was found for the remainder of the comparisons. An association of the other major haplotypes with response to RTX was not found, while the rare haplotypes were more prevalent among the nonresponders (the TTTC haplotype in particular, with 0.109 in nonresponders versus 0.064 in moderate responders versus 0.039 in good responders; *P* not significant [NS]).

We then investigated the link between BLyS haplotypes and response to RTX by distinguishing between seropositive and seronegative RA patients. In fact, the presence of RF and anti-CCP antibodies is a well-established predictor of response to RTX (2–4), and seropositivity versus seronegativity characterizes different subsets of RA based on genetic studies (27–33). Of note, the association between the presence of the TTTT haplotype (either in homozygosis or in heterozygosis) and response to RTX was significant only in the seropositive subset and when comparing good response with absence of response, excluding moderate responders. Of seropositive patients carrying at least 1 TTTT haplotype, 18 of 49 (36.7%) were good responders, 21 of 70 (30%) were moderate responders, and 1 of 13 (7.7%) were nonresponders (for good responders versus nonresponders, OR 6.97 [95% CI 0.84–58.14], $P = 0.050$). The remainder of the comparisons were not significant.

In contrast, no association between BLyS haplotypes and response to RTX was noted in the subset of seronegative RA patients, where good responders (2 patients) and moderate responders (8 patients) were grouped together and compared with nonresponders (10 patients) due to the limited number of patients. Also, the study of the single –871 BLyS polymorphism did not reveal a significant association in this subset, although a higher rate of response (good or moderate) was observed in CC patients than in CT/TT patients (5 of 6 [83.3%] versus 5 of 14 [35.7%]) (OR 9 [95% CI 0.81–100.20], $P = 0.14$). Thus, BLyS genetic variants may be differently associated with response to RTX in seropositive compared to seronegative RA.

In seropositive RA patients, a further subanalysis was done distinguishing between patients who did and patients who did not receive anti-TNF therapies before receiving RTX (2). In seropositive patients in whom anti-TNF therapy had failed, the TTTT haplotype was present only in responders. In fact, 10 of 31 TTTT carriers were good responders (32.3%), 13 of 52 were moderate responders (25%), and 0 of 11 were nonresponders (0%) (for good responders versus nonresponders, OR 11.2 [95% CI 0.60–209.67], $P = 0.04$). The remainder of the comparisons were not significant. Thus, the presence of at least 1 TTTT haplotype in seropositive RA patients in whom anti-TNF agents had failed was associated with a good response to RTX rather than an absence of response. In contrast, since seropositive RA patients receiving RTX as first-line therapy did not respond to RTX very rarely (2 of 38

[5.3%]), a statistical evaluation of BLyS genetics as a predictor of response to RTX could not be performed.

**Replication study.** The replication study was conducted in 117 RA patients from 8 independent rheumatology centers (7 in Italy, 1 in the UK). The demographic and clinical features of these patients are illustrated in Table 1. The patients in the replication study were comparable to those in the first study population in terms of age, sex, disease duration, and baseline disease activity (mean ± SD DAS28-ESR 5.85 ± 1.31 versus 5.91 ± 1.11, respectively; *P* NS). As illustrated in Table 1, the percentages of seropositive patients (RF positive and/or anti-CCP positive) were comparable (82.9% in the replication study versus 86.8% in the first study population; *P* NS), although fewer patients in the replication study were RF positive (69.2% versus 80.9%; OR 1.81 [95% CI 1.03–3.19], $P = 0.0437$). Of note, more patients in the replication study were anti-TNF naive (50 of 117 [42.7%] versus 40 of 152 [26.3%]; OR 2.09 [95% CI 1.25–3.50], $P = 0.0061$).

The overall rate of response to RTX was comparable to that found in the first study patients (32 of 117 good responders [27.4%], 58 of 117 moderate responders [49.6%], and 27 of 117 nonresponders [23.1%]). Seropositive (RF positive and/or anti-CCP positive) patients again showed a higher response than seronegative patients (82 of 97 [84.5%] versus 8 of 20 [40%]; OR 14.35 [95% CI 5.37–38.36], $P < 0.0001$), and anti-TNF–naive patients tended to respond better (82%) than patients in whom anti-TNF therapy had failed (73.1%) (*P* NS).

The genotypic prevalence of the –871 C>T BLyS polymorphism was similar in both the first and replication populations. Again, no association was found between the –871 BLyS polymorphism and response to RTX (data not shown).

BLyS haplotypes were then investigated in seropositive RA patients, and we distinguished patients in whom anti-TNF agents had failed from patients who were anti-TNF naive. The association between the TTTT haplotype and good response to RTX was confirmed in seropositive RA patients in whom anti-TNF agents had failed, since good responders to RTX showed the highest TTTT cumulative frequencies (0.375 in good responders versus 0.145 in moderate responders versus 0.050 in nonresponders) and the highest percentage of patients carrying at least 1 TTTT haplotype (8 of 12 [66.7%] in good responders versus 7 of 21 [33.3%] in moderate responders versus 1 of 10 [10%] in nonresponders). Statistical analyses revealed the highest significant difference when comparing good responders with nonresponders (for good responders versus nonresponders, OR 18 [95% CI 1.65–196.42], $P = 0.01$ and for good responders versus moderate responders and nonresponders combined, OR 5.75 [95% CI 1.36–24.40], $P = 0.03$). The remainder of the comparisons were not significant. Thus, as in the first study population, the presence of the TTTT haplotype in seropositive RA patients in whom anti-TNF agents had failed was significantly associated with a good response to RTX. Again, no significant association was found between the BLyS haplotypes and response to RTX in seropositive anti-TNF–naive patients or in seronegative RA patients (data not shown).

Finally, the –871 CC genotype tended to be more prevalent in seronegative RA patients who responded to RTX (good/moderate response in 4 of 9 CC patients [44.4%] versus 4 of 11 CT/TT patients [36.4%]; *P* NS) also in the replication study.

**Results by grouping the first series of patients with the replication series.** In the whole series of 147 seropositive RA patients in whom anti-TNF therapy had failed (94 in the first series plus 53 in the replication series), the association between the TTTT BLyS haplotype and good response to RTX reached a higher statistical significance (Figure 2A). The TTTT cumulative frequency was 24.4% in good responders versus 4.59% in moderate responders and 2.49% in nonresponders (for good/moderate responders versus nonresponders, OR 8.6 [95% CI 1.15–64.27], $P = 0.0094$ and for good responders versus nonresponders, OR 12.37 [95% CI 1.59–96.00], $P = 0.0018$) (Figure 2A). Patients carrying at least 1 TTTT haplotype were more prevalent among good responders than among moderate responders (18 of 43 [41.9%] versus 20 of 83 [24.1%]; OR 2.27 [95% CI 1.03–4.99], $P = 0.044$) or among nonresponders (18 of 43 [41.9%] versus 1 of 21 [4.8%]; OR 14.4 [95% CI 1.77–117.39], $P = 0.0028$). Even if the number of cases was limited, no significant association was found between BLyS polymorphisms and response to RTX in the whole series of anti-TNF–naive seropositive patients (Figure 2B).

In the seronegative RA patients overall, no significant association was found between BLyS haplotypes and response to RTX. However, a trend was observed for the –871 C>T BLyS polymorphism (good/moderate response in 9 of 15 –871 CC patients [60%] versus 9 of 25 –871 CT/TT patients [36%]; OR 2.67 [95% CI 0.71–9.95], $P = 0.19$).

Notably, the disease duration did not influence the rate of EULAR responses (median [range] 12 years [2–40 years] for no EULAR response versus 10.5 years

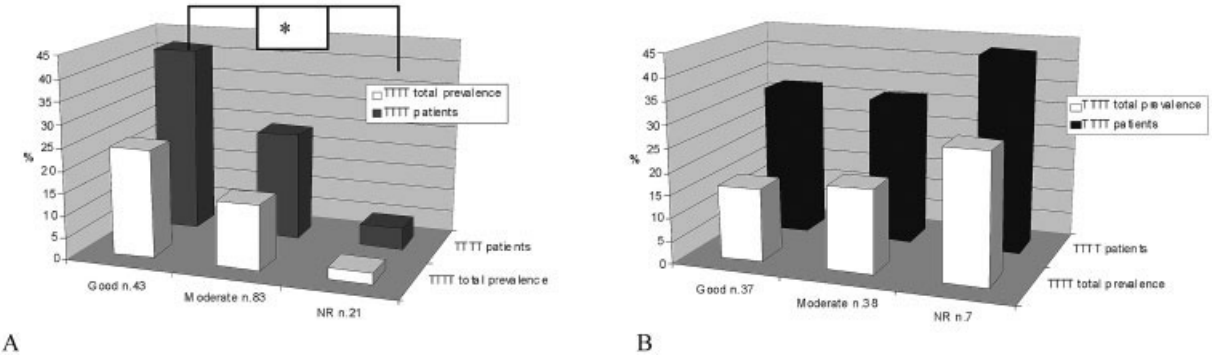


**Figure 2.** The TTTT B lymphocyte stimulator (BLyS) haplotype is associated with response to rituximab (RTX) in seropositive patients in whom anti-TNF agents have failed. **A** and **B,** The cumulative prevalence of the TTTT haplotype among all BLyS haplotypes (open bars) and the percentage of patients carrying the TTTT haplotype (both in heterozygosis and in homozygosis) (solid bars) are represented in the 3 subgroups of response to RTX, both in patients in whom anti-TNF agents have failed (**A**) and in anti-TNF–naive patients (**B**). **A,** In patients in whom anti-TNF agents had failed, the TTTT BLyS haplotype was more prevalent in good responders than in moderate responders or in nonresponders. For good responders versus nonresponders, $*$ = OR 14.4 (95% CI 1.77–117.39), $P = 0.0028$. **B,** No clear association was found in anti-TNF–naive patients. The other BLyS haplotypes are not illustrated since they did not change significantly among the different classes of response to RTX. See Figure 1 for other definitions.

[1–56 years] for EULAR moderate response versus 10 years [1–43 years] for EULAR good response; $P = 0.68$ by Kruskal-Wallis test). Also, the mean ± SD baseline DAS28-ESR was not different among the 3 EULAR response categories (5.6 ± 1.2 for no EULAR response versus 5.9 ± 1.2 for EULAR moderate response versus 6.1 ± 1.1 for EULAR good response; $P = 0.08$ by analysis of variance), and the absence of significant differences among EULAR response categories was also observed for the number of TNF blockers that had previously failed (median [range] 1 [0–4] for no EULAR response versus 1 [0–4] for EULAR moderate response versus 1 [0–3] for EULAR good response; $P = 0.2$ by Kruskal-Wallis test).

**Results of multivariate statistical analyses.** Based on the univariate results, multivariate analyses were performed, using logistic regression analyses in 3 different models, in seropositive RA patients in whom anti-TNF therapy had failed. In the first model, the dependent variable was the presence of a EULAR good response (as the best response between months 4 to 6 after RTX treatment) versus the absence of response; in the second model, the dependent variable was a EULAR good response versus a EULAR moderate response or the absence of response; and, in the third model, the dependent variable was the presence of a EULAR good or moderate response versus the absence of response. Age, sex, baseline Health Assessment Questionnaire (34) score, baseline DAS28-ESR, previous anti-TNF therapy, and the presence of the TTTT BLyS promoter haplotype were introduced as covariates. Of note, the association of the TTTT BLyS haplotype with response to RTX was significant only in the first and second models (OR 16.2 [95% CI 1.7–152.5], $P = 0.01$ and OR 3.1 [95% CI 1.2–7.8], $P = 0.02$, respectively; $P$ NS in the third model). Thus, the association of the TTTT BLyS haplotype with a good response to RTX, rather than with a moderate response, was again highlighted. Conversely, in patients not carrying the TTTT haplotype, there was a higher risk of no response (Figure 3).

**Possible relationship between BLyS serum levels and genetic variants.** Sera collected before RTX therapy were available from 82 RA patients, and BLyS serum levels appeared significantly more elevated in RA patients than in controls (mean ± SD 1,102 ± 484 pg/ml versus 655 ± 158 pg/ml; $P < 0.0001$). Increased BLyS

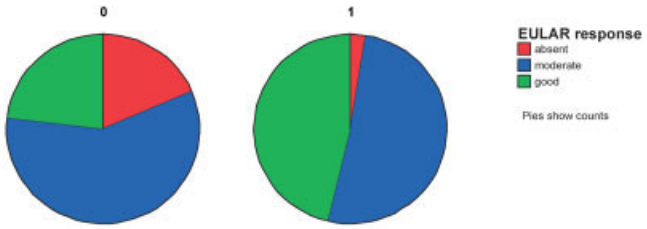


**Figure 3.** Pie charts showing different patterns of European League Against Rheumatism (EULAR) response in the pooled series of seropositive rheumatoid arthritis patients in whom anti–tumor necrosis factor agents had failed. Left, Pattern of EULAR response in patients not carrying at least 1 TTTT haplotype (0). Right, Pattern of EULAR response in patients carrying at least 1 TTTT haplotype (1).

levels (i.e., >939 pg/ml, which represents the 95th percentile, such as the upper cutoff of the physiologic range) were found in 42 of 82 patients (51.2%). In RA, BLyS levels did not correlate significantly with age, but appeared significantly more elevated in females than in males (709 ± 161 pg/ml versus 611 ± 144 pg/ml; $P = 0.0196$). BLyS serum levels were similar in seropositive and seronegative RA (1,083 ± 468 pg/ml versus 1,149 ± 531 pg/ml; $P = 0.64$) and similar in patients in whom anti-TNF agents had failed as compared to anti-TNF–naive patients (1,077 ± 466 pg/ml versus 1,181 ± 564 pg/ml; $P = 0.33$). In contrast to previous data (22), the different –871 C>T BLyS genotypes were not associated with significantly different BLyS serum levels (1,038 ± 506 pg/ml for CC, 1,161 ± 511 pg/ml for CT, 1,007 ± 266 pg/ml for TT; *P* NS).

Among the seropositive RA patients in whom anti-TNF agents had failed, good responders to RTX tended to have lower baseline BLyS levels than moderate responders, who in turn tended to have lower baseline BLyS levels than nonresponders, but the difference was not significant (895 ± 174 pg/ml, 967 ± 236 pg/ml, and 1,199 ± 594 pg/ml, respectively; $P = 0.38$ for good responders versus nonresponders). In the same subgroup of patients, BLyS levels in TTTT carriers (977 ± 194 pg/ml) did not differ compared to those in non–TTTT carriers (1,011 ± 404 pg/ml). Considering the subset of seronegative RA patients, the –871 CC patients tended to show higher BLyS serum levels than the CT/TT patients (1,341 ± 730 pg/ml versus 1,026 ± 328 pg/ml; $P = 0.24$), but responder patients did not have higher BLyS serum levels than nonresponder patients (1,138 ± 471 pg/ml versus 1,156 ± 583 pg/ml; *P* NS). In conclusion, a significant relationship between BLyS polymorphisms and BLyS serum levels was not found in this study.

## DISCUSSION

In the present study, we investigated the association between BLyS genetics and response to RTX in RA, extending for the first time in RA, the genetic study to all the 4 common polymorphisms located in the BLyS promoter (23). We found a significant association between BLyS genetics and good, rather than moderate, response to RTX. The results were confirmed by replication studies. In a first series of 152 RA patients, we found a significant association between the TTTT BLyS haplotype and a good response to RTX, and this association was limited to seropositive RA patients in whom anti-TNF therapy had previously failed. Data were then confirmed in a second independent series of 117 RA patients, and pooled analyses on the overall cases showed an even stronger relationship between a good response to RTX and the TTTT BLyS haplotype.

The list of biologic agents for RA is rapidly growing, and the identification of genetic biomarkers able to predict response to therapy is a relevant issue (27). However, previous data in the literature were often preliminary and replications were frequently lacking or had conflicting results. Moreover, different subsets of patients were not analyzed in detail. In the present study, the replication series was fundamental to confirming the TTTT BLyS haplotype as a specific marker of good response to RTX in a definite subgroup of RA patients.

Although RTX may prove effective as first-line biologic therapy (28), as was also observed in the present study, this drug is licensed for use only after the failure of anti-TNF agents in RA. Second, RTX is recommended more often in autoantibody-positive RA patients. Third, a good response to therapy, rather than a moderate response, is currently the main goal of any biologic therapy in RA (29). Thus, the TTTT BLyS haplotype, the reported genetic marker associated with a good response to RTX in seropositive RA patients in whom anti-TNF therapy has previously failed (2), is of clinical relevance.

The response to anti-TNF therapy may be a tool by which to identify a subset of RA in which TNF may be relevant for treatment of chronic synovitis (30). Based on the present results, the failure of anti-TNF therapy may also highlight a subset of RA in which B cells may be more implicated and in which BLyS genetic studies are more helpful.

In addition, the present study compared seropositive (RF-positive and/or anti-CCP–positive) RA patients (n = 229), who are widely recognized as the best responders to RTX (2–5), to seronegative RA patients (n = 40). Although the number of seronegative patients was limited, our findings suggest a different role of BLyS genetics as a biomarker in either seropositive or seronegative RA, similar to other genetic biomarkers (31–33,35–38).

BLyS favors B cell survival and proliferation (14), is overexpressed in RA synovium (39,40), and is increased after RTX therapy (20,21), and this might favor resistance to RTX (41,42). The association of BLyS genetics with response to RTX may be linked to a role of BLyS in sustaining the proliferation of autoimmune B cell clones, autoantibody secretion, and chronic RA synovitis before and/or after RTX therapy.

In the present investigation, the –871 C>T BLyS polymorphism was not confirmed as a suitable genetic marker for predicting response to RTX in the overall RA population, in contrast to preliminary data in a smaller series (22). However, the possible usefulness of the –871 C>T BLyS polymorphism in seronegative RA may need further investigation.

Neither the study of the –871 C>T BLyS polymorphism nor the study of the complete BLyS promoter haplotype was sufficient to identify a significant relationship between BLyS serum levels and BLyS genetic variants in RA in the present study. One possible explanation is that the commercial ELISAs used for the BLyS dosage, including the ELISA used in the present study, may be insufficient to give a complete picture of BLyS expression (43,44). Furthermore, membrane expression of which may differ from serum levels of BLyS, can be used to infer the presence of RA disease activity and predict response to RTX. More comprehensive analyses are then required. Our group has planned additional studies of the different BLyS isoforms (43,44) in serum and at the tissue level.

In conclusion, the BLyS promoter haplotype may represent a useful tool with which to predict a response to RTX in those RA patients in whom this drug is currently recommended (i.e., seropositive patients in whom anti-TNF therapy has failed). Of note, we found a relationship between BLyS genetics and a good response to therapy, which is the current goal of biologic treatment.

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## AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. De Vita had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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**Acquisition of data.** Fabris, Quartuccio, Vital, Pontarini, Salvin, Fabro, Zabotti, Benucci, Manfredi, Ravagnani, Biasi, Atzeni, Sarzi-Puttini, Morassi, Fischetti, Bazzicchi, Saracco, Pellerito, Cimmino, Carraro, Semeraro, Schiavon, Caporali, Bortolotti, Govoni, Fogolari, Tonutti, Bombardieri, Emery, De Vita.

**Analysis and interpretation of data.** Fabris, Quartuccio, Emery, De Vita.

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