

Rituximab induces a lasting, non-cumulative remodelling of the B-cell compartment

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Abstract

Objective

Reconstitution of B-cells after their therapeutic depletion with rituximab mimics the ontogeny of the B-cell lineage in patients with rheumatoid arthritis. However, little is known about the effects of multiple cycles of treatment on the repletion kinetics and their long-lasting effects on the B-cell compartment. We therefore compared the recovery capacity of the B cell subpopulations between patients who experienced their first cycle of rituximab and those who experienced successive cycles.

Methods

The distribution of the different B-cell subsets was characterised by multiparametric flow cytometry in the peripheral blood of 29 patients in the first rituximab course (naïve cycles) and 40 patients in successive cycles. Samples were obtained at baseline and at 3, 6, and 8 months of each cycle.

Results

The baseline percentage of B-cell subsets was similar among successive cycles. Therefore, successive cycles were grouped for comparison with naïve cycles. Patients in naïve cycles had higher percentages at baseline of both total and memory B-cells. However, the recovery of the different B-cell subsets was similar between naïve and successive cycles. In naïve patients the percentage of transitional B-cells significantly correlated with disease activity at baseline.

Conclusion

Rituximab induces a long-term reshape of the B-cell compartment while multiple cycles of rituximab do not induce cumulative effects on B-cell subpopulations. Transitional B-cells seem to be associated with higher disease activity, although further studies are needed to determine if they can be used as a biomarker to predict the need for rituximab retreatment.

Key words

rheumatoid arthritis, B-lymphocytes, rituximab

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Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disorder characterised by chronic synovitis leading to joint damage and disability, as well as systemic manifestations (1). At present, the pathogenesis of RA is considered to involve the interaction of environmental factors with a genetically predisposed host (2). Recent genetic studies suggest that pathways related with either T-, B-lymphocyte or the innate immune system are associated to the risk of development of this disorder (3).

Despite HLA-DR variants account for 50–60% of the risk of developing RA (4), pointing to a T-lymphocyte mediated disorder, several findings suggest that B-cells play also an important role in the development and progression of RA (5). Among them we can remark that auto-reactive B-lymphocytes are directly responsible for auto-antibody production, and these cells have been also involved in pro-inflammatory cytokine production and antigen presentation to T-lymphocytes contributing to the activation, differentiation and persistence of autoreactive T cells (6, 7).

Immune-modulation with synthetic disease-modifying anti-rheumatic drugs (sDMARD) is the first line of treatment for RA (8). However, when sDMARD fail, treatment of RA patients with biologic DMARD is recommended (8). Among them, rituximab (RTX), an anti-CD20 monoclonal antibody (mAb) that induces B-cell depletion, has proven to improve signs and symptoms of RA, especially in seropositive forms of the disease (9).

RTX administration induces a depletion of B lymphocyte subsets from pre-B-cells to mature B lymphocytes both naïve and memory (5). Since pro-B lymphocytes and plasma cells do not express surface CD20, these cells are not depleted by RTX (5). Therefore, B cell repopulation is allowed and antibody production is maintained by long-life memory plasma cells (5).

Previous studies have analysed the pattern of peripheral blood B lymphocyte repopulation in small populations of patients treated with RTX. As expected new B-cells belong mainly to immature and naïve B-cell subsets (10–12).

However, there is marked heterogeneity in peripheral blood lymphocytes subpopulations in the general population (13). Therefore, our objective was to determine if there are differences in B cell repopulation between patients that experience their first RTX cycle of treatment and those who have experienced successive cycles of treatment on daily clinical practice. In addition, we asked whether successive RTX cycles induce progressive differences in B cell subpopulations.

Material and methods

Patients and study design

This is a prospective study including 69 patients classified as RA according to 1987 American College of Rheumatology (ACR) criteria (14) that were treated with RTX at our hospital from August 2010 to June 2013. We collected information from 114 courses of RTX treatment, of which 29 corresponded to the first RTX course (from now they are called naïve courses or cycles). The remaining RTX courses (85 cycles) corresponded to cycles 2nd to 9th and they are called successive cycles. Some patients contributed with more than one cycle, of which 9 patients contributed with their first and 2nd cycles, and some other patients contributed up to 4 cycles. At statistics section we provide information on how this circumstance was considered in order to avoid bias because of over-representation of the cases with higher number of cycles, as well as whether there was an additive effect of RTX treatment. Regarding the latter, successive cycles were clustered in the following groups: 2nd and 3rd cycles, 4th and 5th, and 6th or higher. This distribution was decided in order to get similar number of cycles in each group, to avoid statistical problems due to underrepresentation of any of the groups.

Disease activity at baseline was assessed through the disease activity score calculated with 28 tender and swollen joint counts (DAS28), as previously described (15).

RTX treatment

At our hospital there is no fixed protocol for RTX use in RA patients and treat-

ment is prescribed based on physician criteria (16). For this study we only included patients treated “on demand” when the disease relapses. So, none of the patients reported in this work received RTX in a fixed schedule every 6 months. Furthermore, in 14 successive cycles, patients were re-treated with RTX despite B-cells were not repopulated in their peripheral blood. These cycles were also excluded from analysis. RTX was administered in 2 infusions of 1000 mg 15 days apart in almost all naïve cycles. However, in 28% of successive cycles only 1000 mg of RTX was administered. This profile of RTX administration, although infrequent in our early experience, was common at the time of conducting this study (16). In addition, 1000 mg RTX cycles are more frequent with increasing number of cycles (16).

Sample collection and flow cytometry analysis

Peripheral blood samples were obtained in EDTA tubes for cytometry assessment. The schedule of sample collection was as follows: at baseline (T0) immediately previous to RTX infusion and then at 3 (T3), 6 (T6), 8 (T8), 10, 12, 14, 16 and 18 months if there was no previous relapse. The median clinical response duration in our population has been described to be 10 months (16), leading to prescription of a new RTX cycle. Thus, available data gradually decreased beyond month 8 precluding an adequate statistical analysis. Therefore, we decided to censor the data analysis at month 8 since at this visit most patients had started B cell repopulation.

For flow cytometry analysis, we first determined absolute number of CD19

positive cells (cells/ μ L) through staining of 100 μ L of whole peripheral blood with phycoerythrin anti-CD19 mAb (Becton Dickinson [BD], San Jose, CA, USA) in TruCOUNT tubes (BD) that contained a known number of fluorescent beads by comparing cellular events to beads events with the software Infinicyt (Cytognos, Salamanca, Spain). When samples had more than 5 B-cells/ μ L, a further immunophenotypic characterisation of the B cell subpopulations was performed using the following mAbs: anti-CD45 (Horizon V500), anti-human IgM (Allophycocyanin [APC]), anti-CD38 (APC), anti-CCR6 (Phycoerythrin [PE]), anti-CD21 (PE), anti-CD27 (peridinin chlorophyll protein [PerCP]–Cy5.5), anti-CD19 (PE–Cy7), anti-human IgD (fluorescein isothiocyanate [FITC]), anti-CD24 (FITC), anti-CD10 (APC–H7), anti-CD38

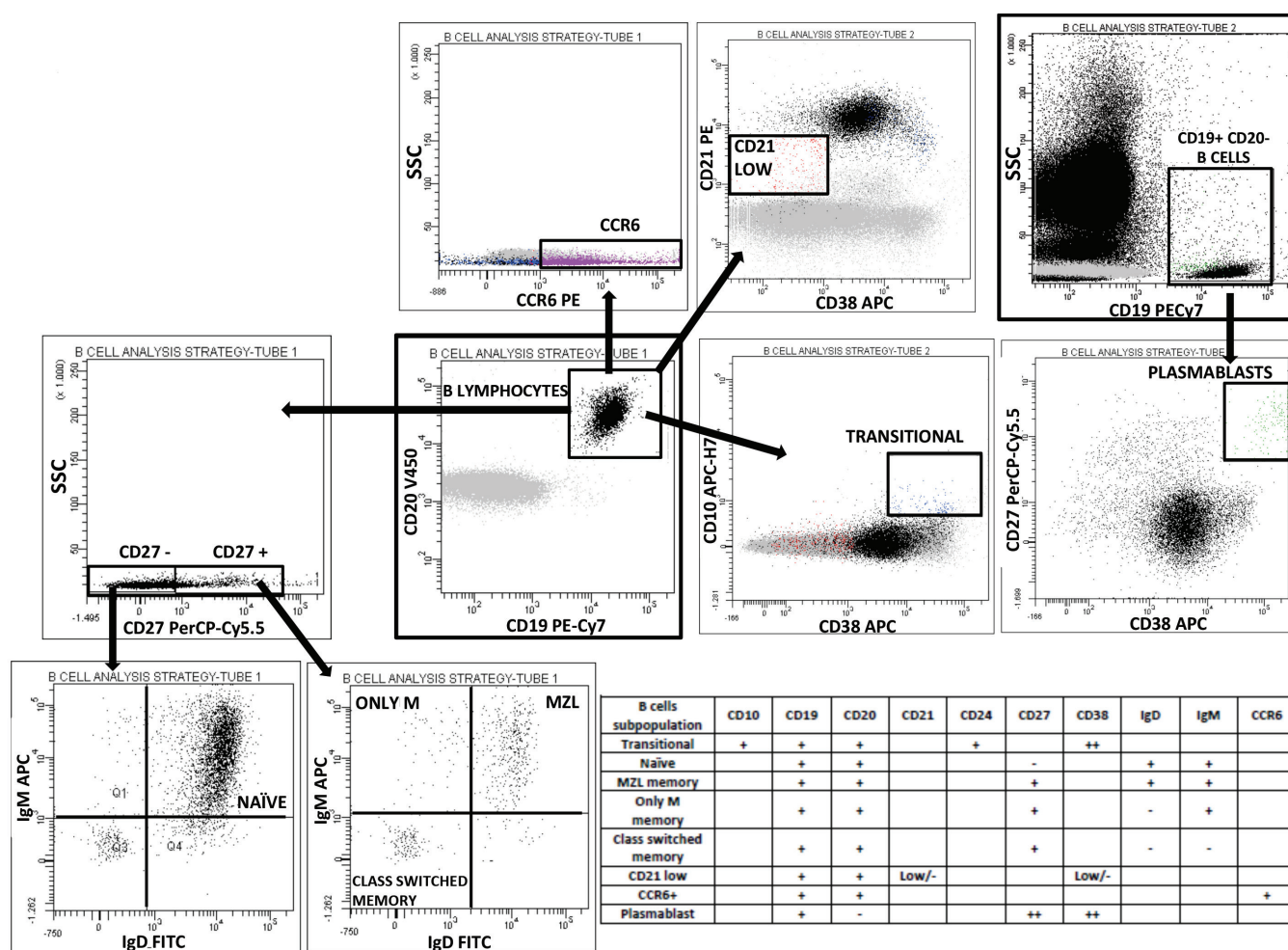


Fig. 1. Gating strategy used for the identification of B-cell subpopulations according to their maturation stage. Bivariate dot-plot graphical representations are shown of the gating strategy used to identify transitional, naïve, marginal zone like, class-switched memory B-cells and plasma cells, according to their pattern of expression of the molecules shown in the table and their side-scatter (SSC) features.

(APC-H7) from BD and anti-CD20 Pacific Blue from BioLegend.

Samples were incubated for 15 minutes at room temperature with the respective mAb. Then erythrocytes were lysed with FACS lysis solution (BD) for 10 minutes and after washing with phosphate buffered saline, samples were analysed in a FACS Canto II device (BD). At least 100,000 cells were collected and data were analysed with the FACSDiva Software (BD). B cell subpopulations were assigned according to the strategy shown in Figure 1.

Statistical analysis

Statistical analysis was performed with Stata v. 13.1 (StataCorp, College Station, TX, USA).

Quantitative variables were represented as the mean \pm standard deviation (SD), since total n was higher than 30 and most of variables fulfilled the homoscedasticity criteria assessed by Levene's test. Differences in these variables were analysed using the Student's *t*-test (successive vs. naïve). When multiple groups existed (different number of successive cycles), they were analysed with the *nptrend* command of Stata that performs the Cuzick's non-parametric test for trend across ordered groups. Categorical variables were expressed as percentages and significance levels between groups were established using the χ^2 test or the Fisher's exact test.

For the statistical analysis of B-cell subpopulations only baseline and 8 month visits were used, since few patients had repopulated at the 3- and 6-month visits. Student's *t*-test was used and since multiple comparisons were performed, the significance threshold was set at $p < 0.005$, according to Bonferroni correction. In addition, considering that we studied repeated measurements (different rituximab cycles) for each patient, a sensitivity analysis was performed using population-averaged panel-data models through the command *xtgee* of Stata. This command allows fitting analysis using generalised estimation equations nested by patient and cycle to adjust for repeated measurements. This approach yielded almost same results than with Student's *t*-test (data not shown).

Table I. Characteristics of the patients according to rituximab cycle groups.

	Naïve cycles (29)	Successive cycles (85)		
		2 nd and 3 rd (28)	4 th and 5 th (32)	6 th or higher (55)
Female gender	26 (90)	25 (89)	28 (87.5)	21 (84)
Age at disease onset (years)	48.5 \pm 15.5	50.9 \pm 12.4	45.0 \pm 9.7	47.7 \pm 13.3
Disease duration (years)	12.0 \pm 8.7	13.1 \pm 6.8	14.0 \pm 6.8	16.8 \pm 6.9
Rheumatoid factor positive	28 (96.5)	28 (100)	30 (94)	23 (92)
DAS28 at baseline	5.1 \pm 1.3	4.9 \pm 0.9	4.8 \pm 1.3	4.6 \pm 1.0

DAS28: disease activity score calculated with 28 joint count (15). The data are shown as mean \pm standard deviation or total number (percentage).

Pearson's test was used to analyse the correlation between disease activity and baseline B-cell subpopulations.

Ethical statements

This is an analysis of a subpopulation of RITuximab en Artritis Reumatoide (RITAR) study that was conducted according to the principles expressed in the Helsinki Declaration of 1983. RITAR was considered by the Spanish Agency of Medicines and Medical Devices (AEMPS) as an observational study (EPA-OD) and it was approved by the Research Ethics Committee of Hospital Universitario La Princesa (code no. 2903A), that did not consider necessary to ask for the written consent of patients. Nevertheless, patients were informed about the study, and verbal consent was obtained.

Results

Rituximab does not induce cumulative effects on peripheral B lymphocyte subpopulations

Before comparing naïve cycles with successive cycles, we studied whether RTX induces cumulative changes in B cell subpopulations in the latter group. Thus, we compared baseline data from patients receiving their successive cycles clustered at the 3 groups described in Table I. As it is shown in Figure 2, we did not observe significant differences in the percentage of the different B cell subpopulations among the 3 groups. The only exception was a non-significant trend to lower percentage of plasmablasts with increasing number of administered RTX cycles. Therefore, we decided to consider all the successive cycles as one group for the subsequent analyses.

Baseline differences in peripheral B lymphocyte subpopulations between naïve and successive patients

As expected, at baseline those patients studied in successive cycles showed significantly lower total B-cells percentages than naïve patients (Fig. 3, upper panel; time=0).

Regarding the percentage of B-cell subpopulations at baseline, patients in naïve cycles showed significantly higher percentages of memory mature B-cells, as well as their subsets: marginal-zone-like B-cells, Class switched B-cells and only M memory B-cells (Fig. 3, 2nd and 3rd rows of panels). By contrast, naïve patients showed significantly lower percentages of transitional B-cells and no significant difference in the percentages of naïve mature B-cells (Fig. 3, left and middle panels of 2nd row). There were no differences in the percentage of plasmablasts and there was a trend to higher percentage of CD21 low and CCR6+ B-cells in peripheral blood of patients at naïve cycles (Fig. 3, 4th row).

Follow-up differences in peripheral B lymphocyte subpopulations between naïve and successive cycles

At the 3-month visit, we observed that 3 patients did not achieve a complete B-cell depletion, one of them after two 2000 mg RTX cycle and the other two patients after 1000 mg RTX cycle.

As previously described (5), the rate of repopulation was heterogeneous starting at 6 months in 20.8% of cases; 55% of patients repopulated at 8 months, 80% at 10 months and almost all patients at 12 months. There were no significant differences in the rate of repopulation between naïve and successive cycles (Fig. 3, upper panel).

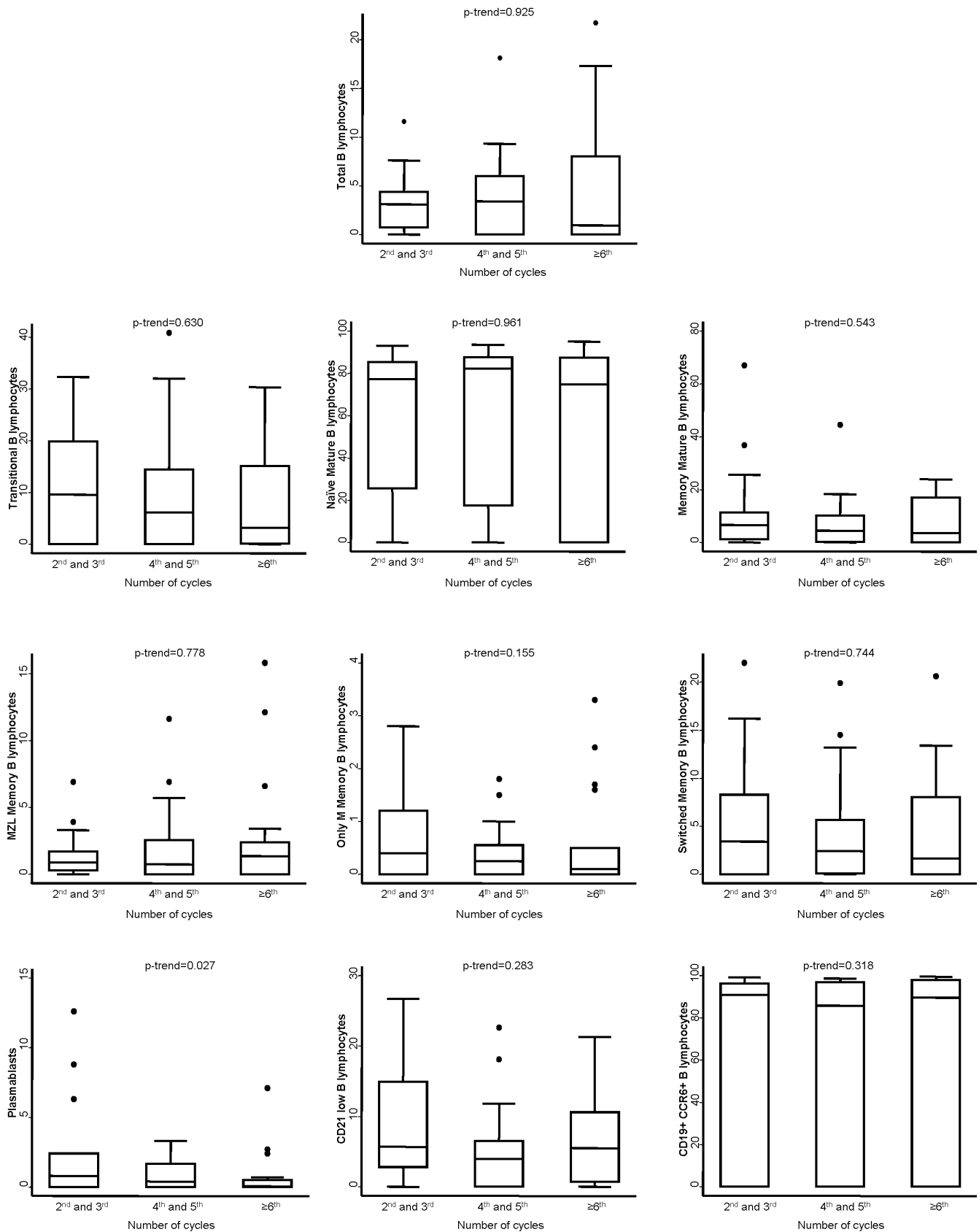


Fig. 2. Differences in B cell subpopulations at baseline visit among patients that had received 2 or more cycles of rituximab. The data represent percentage with respect to total lymphocytes in the first row of panels or percentage with respect to B lymphocytes in all other panels. Data are shown as interquartile range (p75 upper edge, p25 lower edge, p50 midline), p95 (line above the box) and p5 (line below the box). MZL: Marginal zone like. The Cuzick's test for trend was used to determine statistical significance that was set to $p\text{-trend} < 0.005$ due to multiple comparisons, according to Bonferroni correction.

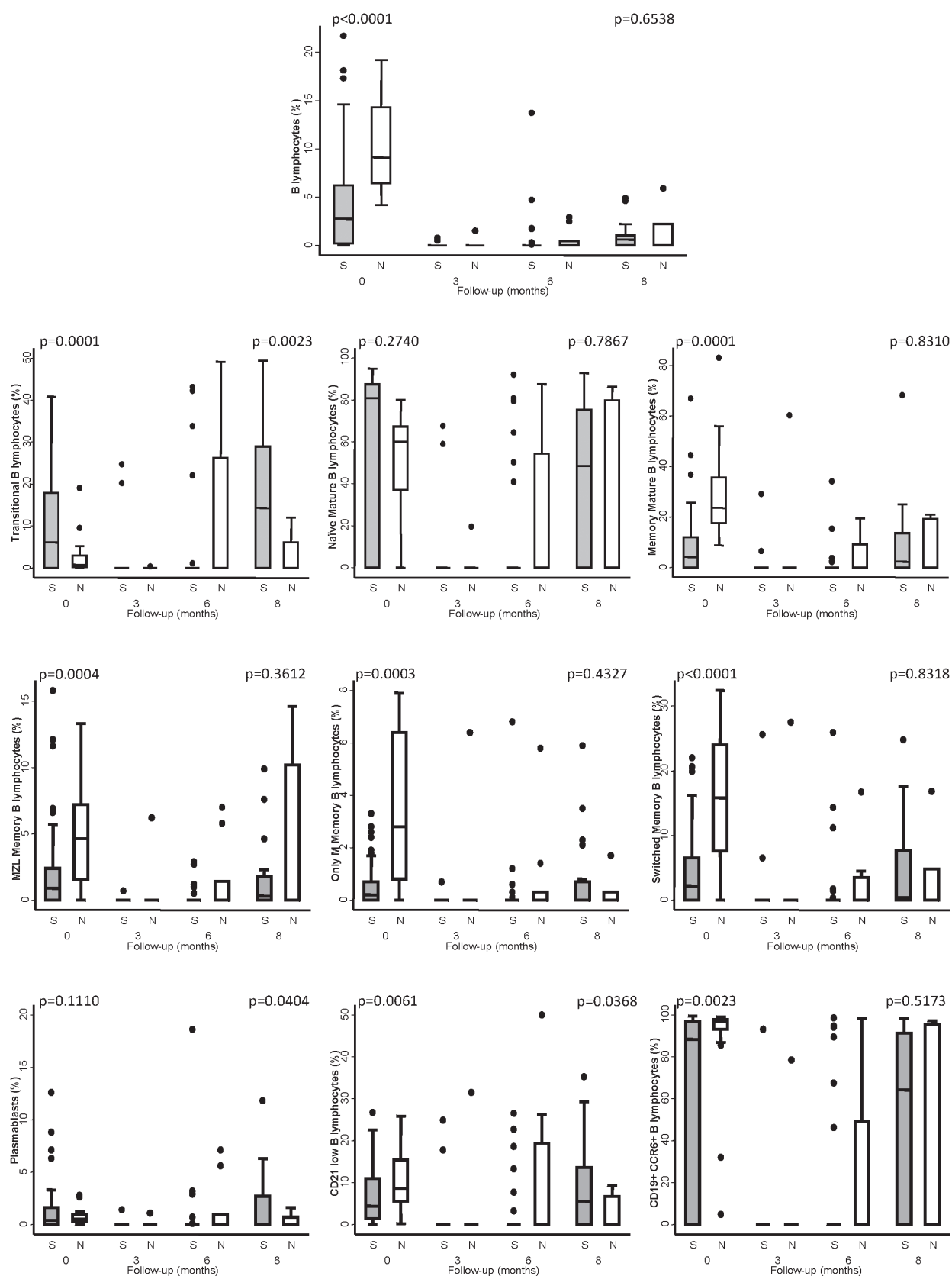


Fig. 3. Comparison of B cell subsets at baseline and repopulation between naïve (N) and successive (S) patients undergoing rituximab treatment. The data represent percentage with respect to total lymphocytes in the first row of panels, or percentage with respect to B lymphocytes in all other panels. Data are shown as interquartile range (p75 upper edge, p25 lower edge, p50 midline), p95 (line above the box) and p5 (line below the box). MZL: Marginal zone like. Statistical analysis was performed at the baseline and 8-month visits when enough data were available (most patients were B-cell depleted at the 3- and 6-month visits) using Student's *t*-test. Significance threshold was set at $p<0.005$ due to multiple comparisons, according to Bonferroni correction.

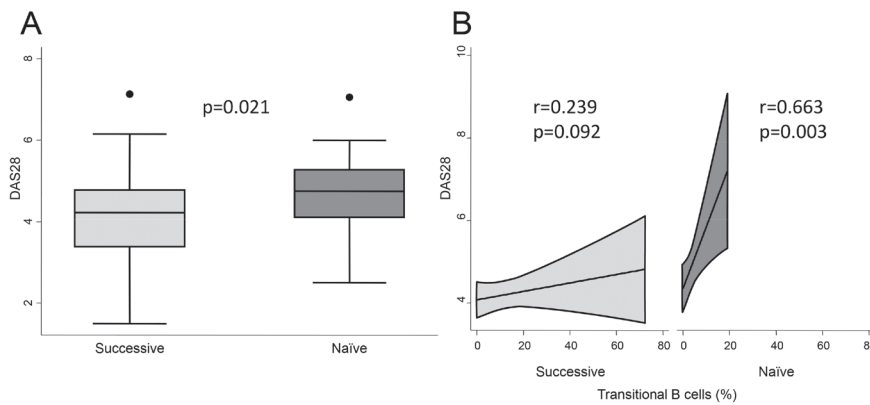


Fig. 4. Transitional B-cells correlate with disease activity in patients with rheumatoid arthritis.

A: Baseline disease activity in patients undergoing their first rituximab cycle (naïve patients, dark grey boxes) or successive (light grey boxes) rituximab cycle. The data are shown as interquartile range (p75 upper edge, p25 lower edge, p50 midline), p95 (line above the box) and p5 (line below the box) of DAS28. Dots represent outliers. Statistical analysis used was Student's *t*-test. Significance threshold was set at $p < 0.05$. **B:** Association between disease activity and percentage of transitional B-cell at baseline. The data are shown as the linear correlations (solid red line) and their respective 95% confidence intervals (grey areas) for successive cycles (left panel) and naïve cycles (right panel). The ρ -coefficient and *p*-level were estimated with the Pearson's test and statistical significance was set at p -level < 0.005 due to multiple comparisons (the complete set of panels justifying this adjustment is shown in Supplementary Fig. 1).

At the 8-month visit, we did not observe differences in most B-cell subpopulations, except for a slightly significant lower percentage of transitional B-cells in patients at their first RTX cycle, that probably is not clinically relevant since at the sixth month the percentage of transitional B-cells was higher in those patients compared to patients receiving successive RTX cycles (Fig. 3, left panel, 2nd row).

Disease activity and B lymphocyte subpopulations

At baseline visit, naïve patients showed significantly more intense disease activity than successive patients (Fig. 4A). Since there were significant differences in several B cell subpopulations at baseline between naïve and successive patients, we studied whether there was any association at baseline between B-cells subpopulations and disease activity. The percentage of transitional B-cells in naïve patients was significantly associated with increased levels of disease activity at baseline (Fig. 4B). In addition, there was a similar trend to significance in successive patients. None of the other subpopulations showed association with disease activity at baseline (Suppl. Fig. 1).

Discussion

Depletion of B-cells by anti-CD20 antibodies like RTX is efficacious in patients with RA (8) and other autoimmune dis-

eases, such as ANCA-associated vasculitis (17, 18) or idiopathic thrombocytopenic purpura (19), underscoring that relevant T-cell driven mechanisms in several autoimmune diseases are B cell dependent. However, both the depletion of B lymphocytes and its therapeutic benefit are transient in most patients since RTX targets the CD20 molecule, which is only expressed by mature B-cells. In addition, B-cell reconstitution after depletion with rituximab usually coincides with a new outbreak of the disease, especially in RA (5).

In this study, we found that successive RTX cycles do not induce a cumulative effect in the percentage of the different B-cell subsets, indicating that B-cell reconstitution always follows a similar pattern after each cycle. We only observed a non-significant tendency to lower percentage of plasmablasts in those patients with a higher number of RTX cycles (Fig. 2, lower row, left panel). Since it has been previously reported that detection of plasmablasts could be associated with disease relapse (10, 11, 20), and best responders are those who received higher number of RTX cycles, it could be argued that plasmablast percentages may decrease with higher number of RTX cycles. However, this was not consistent with the finding of a trend to higher proportion of plasmablasts, either at baseline or after repopulation at

month 8, in patients that received successive cycles compared to naïve patients (Fig. 3, left panel, lower row).

Altogether, our data support previous studies on how B-cells repopulate peripheral blood after depletion with RTX (10, 11, 21). Furthermore, our study gives insight into this topic by including a higher number of reconstitution patterns following B-cell depletion with RTX and analysing a wider array of B-cell subpopulations. As expected, independently on whether the patient has received his/her first or successive RTX cycle, repopulation usually starts after 6–8 months of RTX administration with naïve B-cells. Therefore, at this time treated patients show significantly lower percentages of memory B-cells and higher percentages of naïve B-cells than patients before their first RTX cycle. However, after 8 months of RTX treatment no significant differences are observed between both groups, even among the different subpopulations of memory B-cells (non-switched, only M or switched). This pattern of reconstitution could also be observed by our group in other settings such as bone marrow transplantation (22). Therefore, it constitutes a common synchronised pattern of immune reconstitution after B-cell depletion. The only significant difference found was that patients at their first RTX cycle had significantly lower proportion of transitional B-cells both at baseline and after 8 months of RTX treatment. This finding suggests that immature B-lymphocytes tend to migrate from bone marrow earlier in patients that have experienced successive previous B-cell depletion cycles.

On the other hand, we were interested in studying the effect of RTX in two subpopulations not previously studied: CD21⁺ and CCR6⁺ B-lymphocytes. The percentage of baseline CD21⁺ B lymphocytes was higher in patients that had never been exposed to RTX compared to those in successive cycles. This subpopulation of B-lymphocytes is increased in patients with autoimmune disorders and it is an efficient antigen presenting cell to T-lymphocytes (23). Therefore, decreasing CD21⁺ B-cells could be added to the proposed RTX mechanisms of action in RA. Accordingly, we observed

that disease activity at baseline is lower in patients receiving successive RTX cycles compared with naïve patients (Fig. 4A). However, the design of our study does not allow determining whether the lower percentage of CD21⁺ B-cells in RTX experienced patients is the cause or the consequence of lower disease activity. Furthermore, no correlation was observed between the frequency of this subpopulation and disease activity either in successive or naïve cycles (Suppl. Fig. 1). Regarding CCR6⁺ B-cells, we observed a significantly lower percentage of these cells in RTX experienced patients at the baseline visit of their successive RTX cycles compared to naïve patients. CCR6 expression in memory B-cells is essential to develop a recall response to their specific antigen, since the lack of this molecule seems to prevent a colocalisation with CD4⁺ T cells at the lymphoid follicles (24, 25). In addition, CCR6 and its ligand CCL20 have been involved in the induction of pre-B-cells growth (26). The only subpopulation that shows an association with disease activity is transitional B-cells, which could be in accordance with the higher number of self-reactive B-cells among the circulating peripheral new bone marrow emigrants (27). The clearest correlation was observed in RTX naïve patients and a less clear association in patients receiving successive RTX cycles (Fig. 4B). A previous study suggested that treatment with disease-modifying anti-rheumatic drugs can decrease the percentage of these cells, although it lacked an analysis of their correlation with disease activity (28). Further studies will be needed to determine whether monitoring this B-cell subpopulation could predict clinical relapse and, therefore, the need of a new RTX infusion.

The main limitation of our study is that we did not collect enough information to determine whether the changes in B-cell subpopulations were related with response to RTX, since most of the patients studied were in their successive cycles meaning that they were responders and the proportion of non-responders in naïve cycles was too low to allow such analysis. In summary, our findings support previous studies suggesting that repopulation of B-cells after depletion with RTX

always follows a synchronised pattern with no additive effects on the different subpopulations studied. On the other hand, our data point to a role of CD21^{low} B-cells, CCR6⁺ B-cells and transitional B-cells as the real therapeutic targets of B cell depletion in RA, which warrants further studies.

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