# Targeting the humoral immune system of patients with rheumatoid arthritis

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# Chapter 4

## Residual inflammation after rituximab treatment is associated with sustained synovial plasma cell infiltration and enhanced B-cell repopulation

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# Abstract

## Objective

To investigate the clinical effects of rituximab treatment in relation to immunological effects of rituximab on tissue-derived B-lineage cells and repopulation of circulating B-cells.

## Methods

Twenty-four RA patients were treated with 2x 1000 mgr rituximab and assessed clinically at 4, 12, 18 and 24 weeks using disease activity scores of 44 joints ($DAS_{44}$). Synovial biopsies were analyzed with immunohistochemistry at baseline and 12 weeks after treatment. Peripheral blood mononuclear cells were analyzed by high sensitivity flowcytometry at all timepoints.

## Results

We dichotomized the cohort in patients who achieved a low disease activity ($DAS_{44}$<2.4: LoA-group) and those with persistent disease activity ($DAS_{44}$>2.4: HiA-group) at any time after rituximab treatment. At baseline, the LoA-group had significantly lower $DAS_{44}$-scores (median 3.33 [range 2.84-4.23]) than the HiA-group (median 3.73 [range 3.03-5.23]; p=0.022) and significantly less histological inflammation in synovium (median 6.7 [1-15] versus 16.6 [4-22]; p=0.036). $DAS_{44}$-scores before and after rituximab treatment were associated with synovial infiltration of CD79a+ CD20- B-cells, morphologically resembling plasma cells. Following treatment with rituximab, the LoA-group had significantly reduced repopulation of circulating pre-switched IgD+ B-cells (median 0.044% [range 0.002-0.66] versus 0.45% [range 0.07-9.47]; p=0.006) and post-switched CD27+ B-cells (median 0.17% [range 0.04-0.39] versus 0.67 [0.08-2.05]; p=0.005) compared to the HiA-group.

## Conclusion

The present study demonstrated that a low disease activity state following rituximab was associated with reduced infiltration of CD79a+ CD20- plasma cells in synovium and reduced B-cell repopulation.

# Introduction

Several studies have demonstrated the safety and efficacy of rituximab, a chimeric monoclonal antibody directed at the CD20 membrane protein present on B-cells, for the treatment of patients with rheumatoid arthritis (RA) failing TNF-blocking therapy[1-3]. The benefits of B-cell depletion in RA and the finding of disease-specific autoantibodies prior to clinical manifestations of RA[4,5], have renewed interest in the role of B-cells in RA. However, despite the promising results in clinical trials, the mechanism through which B-cell depletion interferes with pathologic disease processes is still largely unresolved. After a single treatment course of 2 infusions of 1000 mg rituximab, B-cells are reportedly depleted for 6-8 months from peripheral blood[6]. However, when B-cells return after rituximab treatment, the clinical course of patients is unpredictable as some patients experience worsening of disease symptoms whereas others show a prolonged response independent of the reappearance of B-cells in the circulation[7]. A recent report on 24 RA patients showed that 11 (46%) patients experienced a relapse at the time of B-cell return while 13 (54%) patients had a long-lasting response after rituximab treatment despite B-cell return[8]. Explanations for these yet unexplained results involving rituximab treatment may be derived from studies investigating tissue effects in rituximab treated RA patients. In these studies, rituximab led to the depletion of CD20+ B-cells not only in peripheral blood but also in bone marrow and synovium[9-11]. Moreover, the study from our group demonstrated that high pretreatment expression of B-cell markers in synovium predicted non-responsiveness to rituximab treatment[9]. These data confirmed that tissue-derived B-cells are likely to be important to understand the pathological role of B-cells in RA.

The present study aimed to expand the previously reported results on tissue-derived B-cells in a cohort of rituximab-treated RA patients, who failed TNF-blocking therapy. The main goal of the present study was to dissect the relationship between clinical and immunological effects of rituximab treatment on tissue-derived B-lineage cells as well as on repopulating B-cells in the peripheral blood.

# Patients & Methods

## Patients

The present study involved 16 female and 8 male patients (median age 54 [range: 34-82], median disease duration 13 [range: 3-53]) with severe, erosive RA (median $DAS_{44}$ at baseline 3.66 [range: 2.84-5.23]) who participated in a single-center, open-label trial to investigate the clinical and immunological effects of treatment with rituximab, as previously described[9]. An additional patient was excluded from the current analysis because the $DAS_{44}$ at baseline was lower than 2.4 and consequently the clinical effects of rituximab treatment too marginal. All patients had failed treatment with combination(s) of DMARDs and TNF-blocking agents. Patients were treated with rituximab administered as 1000 mg intravenous infusion on days 1 and 15. As premedication, 100 mg methylprednisolone and 2 mg clemastine were administered intravenously and 1000 mg acetaminophen orally. TNF-blocking agents were discontinued with a wash-out period of 8 weeks, whereas DMARDs were continued at the same dosage (methotrexate 2.5-25mg/wk in 21 patients, prednisolone 5-20mg/day in 9 patients and leflunomide 20mg/day in 1 patient). The follow-up was 24 weeks. The study was approved by the Ethics Committee of the Leiden University Medical Center and all patients provided written informed consent.

## Clinical effectiveness

Clinical effectiveness was assessed by the disease activity score of 44 joints ($DAS_{44}$), performed by one research nurse [12]. Patients with high disease activity versus low disease activity were identified using a cut-off for the $DAS_{44}$ of 2.4, equal to the cut-off for high versus low disease activity according to the EULAR response criteria[13]. Patients who achieved a low disease activity at any time in 24 weeks after rituximab treatment (LoA-group) were compared to patients with persistent high disease activity after rituximab treatment (HiA-group). The median time to achieving the lowest $DAS_{44}$ was 18 weeks (range: 4-24) in the LoA-group and 18 weeks (range: 12-24) in the HiA-group ($p=0.75$).

## Sample collection

Heparinized blood samples were obtained at baseline and at 4, 12 and 24 weeks, and when possible also at 18 weeks, after initiation of B-cell depleting therapy.

Bone marrow aspirates were obtained at baseline. Peripheral blood mononuclear cells (MNCs) and bone marrow mononuclear cells (BMMCs) were isolated by density gradient centrifugation over Ficoll-amidotrizoaat (LUMC, Leiden, The Netherlands).

## Arthroscopy, synovial tissue sampling and immunohistochemical analysis

Arthroscopy of clinically affected knees was done at baseline in all patients as previously described[14] and the procedure was repeated at 12 weeks after the first infusion of rituximab. Repeat biopsies at 3 months were obtained from the same knee, unless it had been previously injected with prednisolone in which case the contralateral knee was taken. At each occasion 16-20 pieces of synovial tissue were collected using a 2.0 mm grasping forceps (Storz, Tuttlingen, Germany) and embedded in paraffin until analysis. Immunohistochemical methods and analysis were previously described[9]. Inter-observer Pearson's correlation coefficients were 0.97 for CD20cy, 0.94 for CD79a, 0.80 for CD68, 0.91 for Ki-67, 0.89 for CD138 and 0.92 for CD3 (all p-values < 0.001).

## High sensitivity flowcytometric analysis

Up to $5x10^5$ freshly isolated MNCs were immediately stained by incubating with mouse anti-human monoclonal antibodies (mAbs) in phosphate-buffered saline (PBS)/1,0% bovine serum albumin (BSA) at 4°C for 30 minutes and analyzed by flowcytometry. To increase the sensitivity of our analysis[15], $2\text{-}3x10^5$ events were collected and analyzed whereby 0.05% (lymphogate) corresponded to 55 ± 7 events (or approximately $1.56x10^6$ cells/L), with nil events in isotype controls. The following mAbs were used and titrated to determine the optimal concentration: anti-CD20 FITC (clone 2H7); anti-CD19 PE (clone H1B19); anti-CD27 PE (clone L128); anti-CD19 PerCp-Cy5.5 (clone SJ25C1); anti-CD38 PerCp-Cy5.5 (clone HIT-2); anti-IgD (IAG-2); anti-CD3 APC (UCHT1); anti-CD4 fluorescein isothiocyanate (FITC) mAb (clone RPA-T4); anti-CD8 PE (clone RPA-T4) (all from Becton Dickinson, San Jose, CA). After incubation cells were fixed in 1% paraformaldehyde (LUMC, Leiden, The Netherlands) and analyzed within 24-48 hrs. All stained cells were analyzed with a FACSCalibur (Becton Dickinson, San Jose, CA) flowcytometer and the associated software program FlowJo (Tree Star Inc., Ashland, USA) was used to calculate frequencies within the lymphocyte population. Absolute numbers of CD3+/CD4+ and

CD3+/CD8+ T-cells were obtained by adding TruCount beads (Becton Dickinson, San Jose, CA) to the stained cells, from which a multiplication factor was obtained for individual patients at separate timepoints, in order to calculate the absolute number of B-cells and its subsets. B-cells were defined as CD3-/CD19+ cells, the pre-switched, naïve subset as CD3-/CD19+/IgD+ (referred to as 'naïve B-cells') and the post-switched, memory subsets as CD3-/CD19+/CD27+ (referred to as 'memory B-cells').

### Statistical analysis

Non-parametric Mann-Whitney U tests were used to compare variables ($DAS_{44}$ scores, percentages of B-cells and scores of cellular infiltration in synovium) between two groups of RA patients, HiA- versus LoA-group. Univariate linear regression analysis was used to correlate the ratio of naive and memory B-cells with the percentage of CD19+ B-cells. All analyses were confirmed using $DAS_{28}$ scores, which did not change the results reported. P-values were considered significant when $p \leq 0.05$.

## Results

### Patients with a low B-cell load in synovium achieve low disease activity after a single course of rituximab

We compared the baseline characteristics between patients who achieved a low disease activity (LoA-group: $DAS_{44}$ <2.4[13]) and those who still had a high disease activity (HiA-group: $DAS_{44}$ >2.4[13]) after rituximab treatment.

As shown in Table 1, both groups were comparable with respect to age, sex distribution and disease duration. However, the LoA-group had significantly lower $DAS_{44}$ scores (median 3.33 [range 2.84-4.23]) than the HiA-group (median 3.73 [range 3.03-5.23]; p=0.022) at baseline. Moreover, the LoA-group had significantly lower infiltration of CD20+ (median 0 [range 0-3] versus 2.5 [0-4]; p=0.005), CD79a+ (median 0.67 [range 0-2] versus 2.5 [0.33-4]; p=0.004), CD138+ (median 0 [range 0-2] versus 2.5 [0-4]; p=0.011), CD3+ (median 1 [range 0-4] versus 3.5 [1-4]; p=0.029) and Ki-67+ cells (median 0 [range 0-3] versus 3 [0-4]; p=0.016). Also the overall inflammation score in synovium was significantly lower in the LoA-group (median 6.7 [1-15] versus 16.6 [4-22]; p=0.036). Interestingly, the LoA-group was not different from the

HiA-group with respect to the proportions of CD19+ B-cells in peripheral blood and bone marrow, including pre-switched (IgD+) or post-switched (CD27+) subsets (Table 1). Although proportions of B-cells from the total lymphocyte population are better comparable between peripheral blood and bone marrow, absolute numbers of B-cells and their subsets were also calculated and showed no significant differences (Table 1). These data extended our previous observation that rituximab was particularly effective in patients with low $DAS_{44}$ scores, and low synovial inflammation, at baseline.

## Disease activity after rituximab treatment is associated with CD79a+ CD20- plasma cells in synovium

To expand the baseline findings, we compared synovial tissue infiltrates between patients in the LoA-group and HiA-group *after* rituximab treatment. We found that only CD79a+ residual B-cells, the majority being CD20-, were significantly lower in the LoA-group (median 0.33 [range 0-1.33]) compared to the HiA-group (1.33 [range 1-4]; p=0.016) (Table 2). A trend to significance was observed for infiltrating CD3+ T-cells (median 2 [range 1-4] versus 4 [range 2-4]; p=0.06). Importantly, we noticed that the residual CD79a+ cells in synovium showed a typical morphology of plasma cells, with a large nucleus, an expanded Golgi system resulting in a perinuclear halo and an expansion of cytoplasm (Figure 1). In addition, despite significant baseline differences between the LoA- and HiA-group, there was no difference in the infiltration of CD138+ plasma cells after rituximab treatment (both median 0 [range 0-3]; p=0.91). However, we did find a significant and strong correlation between the reduction of CD138+ plasma cells and the reduction of CD3+ T-cells after rituximab (r=0.62; p=0.014). Taken together, these data indicated that synovial infiltration of CD79a+ CD20- plasma cells was related to disease activity both before and after rituximab treatment.

## Low disease activity after rituximab treatment is associated with reduced B-cell repopulation

Because of the apparently pivotal role of synovial infiltration of CD79a+ CD20- plasma cells, we hypothesized that the persistence of CD79a+ plasma cells in the HiA-group was due to increased differentiation and proliferation of B-cells. To test the latter hypothesis, we examined the repopulation of B-cell subsets in peripheral blood after rituximab expecting increased B-cell levels in patients of

the HiA-group. By using high sensitivity flowcytometry, we observed that the frequencies of repopulating CD19+ B-cells correlated strongly with the ratio between pre-switched (IgD+) B-cells and post-switched (CD27+) B-cells ($r=0.66$; $p<0.001$) (Figure 2). These data indicated that early after rituximab-mediated B-cell depletion the repopulation of circulating B-cells was dominated by post-switched (CD27+) B-cells and thereafter surpassed by reconstitution of pre-switched (IgD+) B-cells.

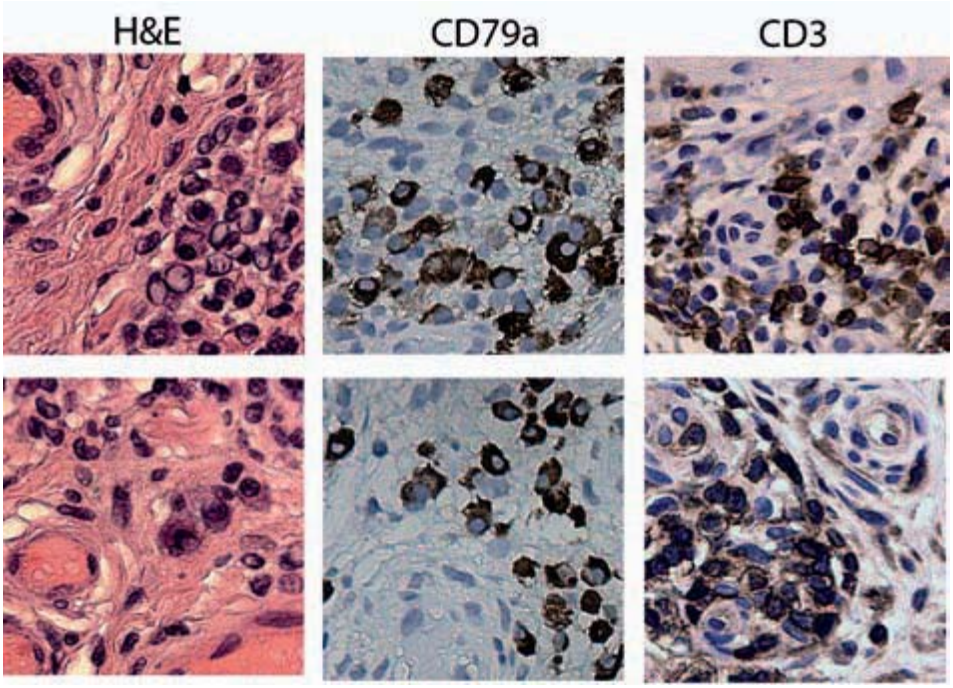


**Figure 1** Residual CD79a+ B-cells after rituximab have a plasma cell morphology. The left panels show H&E (hematoxylin & eosin) staining of plasma cells in synovium of two different patients 12 weeks after rituximab. Typical morphology of plasma cells can be observed: large nucleus compared to surrounding lymphocytes, an expanded Golgi system resulting in a perinuclear halo and an expanded cytoplasm compared to lymphocytes. The middle panels show the morphology of CD79a+ cells of two patients typically resembling plasma cells. For comparison, the right panels show the morphology of CD3+ T-lymphocytes in two patients

**Table 1** Baseline characteristics of patients who achieved low disease activity ($DAS_{44}$ < 2.4) after rituximab treatment (LoA-group) or did not (HiA-group: $DAS_{44} \geq 2.4$). Depicted are median [range]

| | LoA-group (N=11) | HiA-group (N=13) | p-value |
|---|---|---|---|
| **Demographic characteristics** | | | |
| Age (yrs) | 55 [34-82] | 49 [39-65] | 0.17 |
| Female sex (%) | 4 (36%) | 2 (15%) | 0.14 |
| Disease duration (yrs) | 14 [3-53] | 9 [1-32] | 0.40 |
| **Clinical characteristics** | | | |
| $DAS_{44}$ score | 3.33 [2.84-4.23] | 3.73 [3.03-5.23] | **0.022** |
| Tender joints | 9 [6-14] | 11 [6-21] | 0.38 |
| Swollen joints | 3 [1-6] | 8 [3-20] | **0.002** |
| CRP (mg/L) | 27 [2-94] | 29 [2-97] | 0.82 |
| BSE (mm/hr) | 32 [18-109] | 50 [4-114] | 0.09 |
| VAS well-being* | 52 [24-91] | 49 [16-85] | 0.90 |
| **Flowcytometry Peripheral blood** | | | |
| CD19+ (%)† | 5.30 [1.93-14.9] | 9.27 [2.03-18.4] | 0.22 |
| *(cells/L)¶* | *146 [20-494]* | *269 [33-657]* | *0.55* |
| CD19+IgD+ (%)† | 4.15 [0.60-8.28] | 4.57 [1.52-15.4] | 0.39 |
| *(cells/L) ¶* | *97 [6-379]* | *165 [27-339]* | *0.69* |
| CD19+CD27+ (%)† | 2.20 [0.71-4.55] | 2.36 [0.33-6.13] | 0.35 |
| *(cells/L) ¶* | *53 [7-245]* | *64 [5-302]* | *0.74* |
| **Flowcytometry Bone Marrow** | | | |
| CD19+ (%)† | 7.80 [2.90-13.5] | 7.33 [2.03-24.9] | 0.86 |
| CD19+IgD+ (%)† | 5.77 [0.61-10.9] | 5.09 [0.70-21.2] | 0.78 |
| CD19+CD27+ (%)† | 2.09 [0.88-3.67] | 2.11 [0.61-5.57] | 0.83 |
| **Histology Synovium** | | | |
| CD20+ | 0 [0-3] | 2.5 [0-4] | **0.005** |
| CD79a+ | 0.67 [0-2] | 2.5 [0.33-4] | **0.004** |
| CD138+ | 0 [0-2] | 2.5 [0-4] | **0.011** |
| CD68+ | 2.5 [1-4] | 4 [1-4] | 0.065 |
| CD3+ | 1 [0-4] | 3.5 [1-4] | **0.029** |
| Ki-67+ | 0 [0-3] | 3 [0-4] | **0.016** |
| Lining hyperplasia | 1.9 [1-3] | 1.9 [1-3] | 0.65 |
| Overall inflammation | 6.7 [1-15] | 16.6 [4-22] | **0.036** |

* Visual analogue scale for well-being subjectively scored by the patient

† Percentages are analyzed within the lymphocyte gate

¶ To allow comparison of results between all compartments, the percentages of circulating B-cells are shown.

**Table 2** Semiquantitative scores of cellular infiltration in synovial specimens from RA patients 12 weeks after treatment with rituximab. Patients were dichotomized in two groups: those who achieved low disease activity ($DAS_{44} < 2.4$) after rituximab treatment (LoA-group) and those who did not (HiA-group: $DAS_{44} \geq 2.4$). Depicted are median [range].

| Post-treatment | LoA-group (N=11) | HiA-group (N=13) | p-value |
|---|---|---|---|
| CD20+ | 0 [0-1]* | 0 [0-3]* | 0.93 |
| CD79a+ | 0.33 [0-1.33] | 1.33 [1-4] | **0.016** |
| CD138+ | 0 [0-3] | 0 [0-3] | 0.91 |
| CD68+ | 2 [2-4] | 4 [1-4] | 0.43 |
| CD3+ | 2 [1-4] | 4 [2-4] | 0.06 |
| Ki-67+ | 1 [0-3] | 2 [0-4] | 0.23 |
| Lining hyperplasia | 1.67 [1-3] | 1.83 [1-3] | 0.79 |
| Overall inflammation | 7.17 [0-17] | 12.8 [7-20] | 0.06 |

* All patients were negative for CD20cy staining, except for one patient in each group

In addition, we observed that at 24 weeks after rituximab treatment the LoA-group had both significantly lower pre-switched (IgD+) B-cells (median 0.044% [range 0.002-0.66]) as well as post-switched (CD27+) B-cells (median 0.17% [range 0.04-0.39]) in the peripheral blood as compared to the HiA-group (0.45% [range 0.07-9.47] and 0.67 [0.08-2.05], respectively; p=0.006 and p=0.005) (Figure 3). Accordingly, at 24 weeks the LoA-group had significantly fewer circulating B-cells (median 0.25% [range 0.08-1.10]) than the HiA-group (1.08% [range 0.13-9.32]; p=0.013). Importantly, no significant differences in circulating B-cells of its subsets were found at 4 weeks shortly after rituximab treatment. Altogether, these data supported the hypothesis that achieving low disease activity after rituximab treatment was associated with reduced repopulation of B-cells and its subsets, indicative of decreased B-cell proliferation and differentiation.

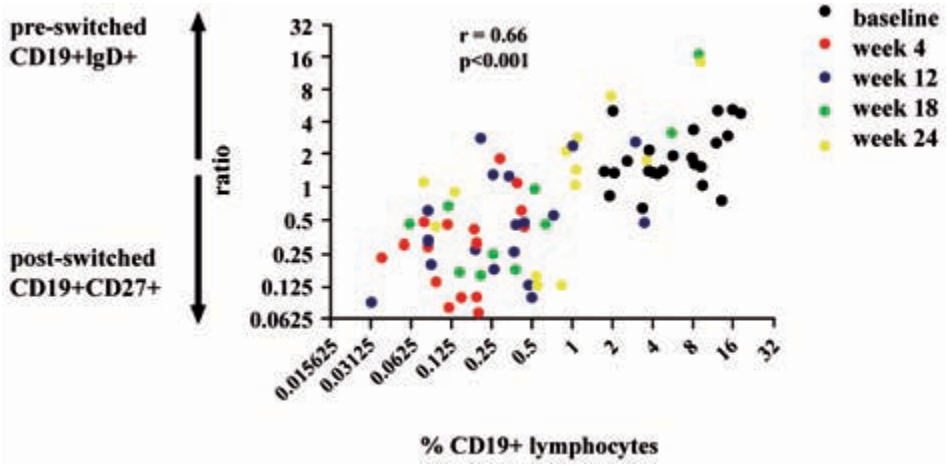


**Figure 2** Repopulation of post-switched B-cells in peripheral blood precedes reconstitution of pre-switched B-cells. The ratio of pre-switched (IgD+) B-cells over post-switched (CD27+) B-cells correlated with the total percentage of circulating B-cells before and after rituximab treatment. To determine the dominant phenotype of B-cells a ratio of CD19+ IgD+ (naïve) B-cells over CD19+ CD27+ (memory) B-cells was calculated with a ratio>1.0 indicating a predominance of CD19+IgD+ pre-switched B-cells and a ratio<1.0 a predominance of CD19+CD27+ post-switched B-cells. Each color of dots represents a different timepoint during the study.

# Discussion

The aim of the present study was to investigate the relationship between clinical effects of rituximab treatment in relation to immunological effects of rituximab on tissue-derived B-lineage cells as well as repopulating B-cells in the peripheral blood. We observed that attaining a low disease activity following rituximab treatment (LoA-group) was associated with reduced synovial infiltration of CD79a+ CD20- plasma cells. Moreover, a significantly slower repopulation of B-cells was observed in patients of the LoA-group, indicating reduced B-cell proliferation. Collectively, the present study demonstrated that rituximab led to low disease activity in patients who had reduced B-cell proliferation together with reduced infiltration of early plasma cells in synovium.

The present study extended previously published data on the predictive value of synovial B-cell load and clinical response to rituximab treatment in RA[9]. Although it seemed counterintuitive that B-cell depleting therapy was the least effective in RA patients with the highest load of synovial B-cells, the present study demonstrated that the presence of CD79a+ plasma cells in synovium was

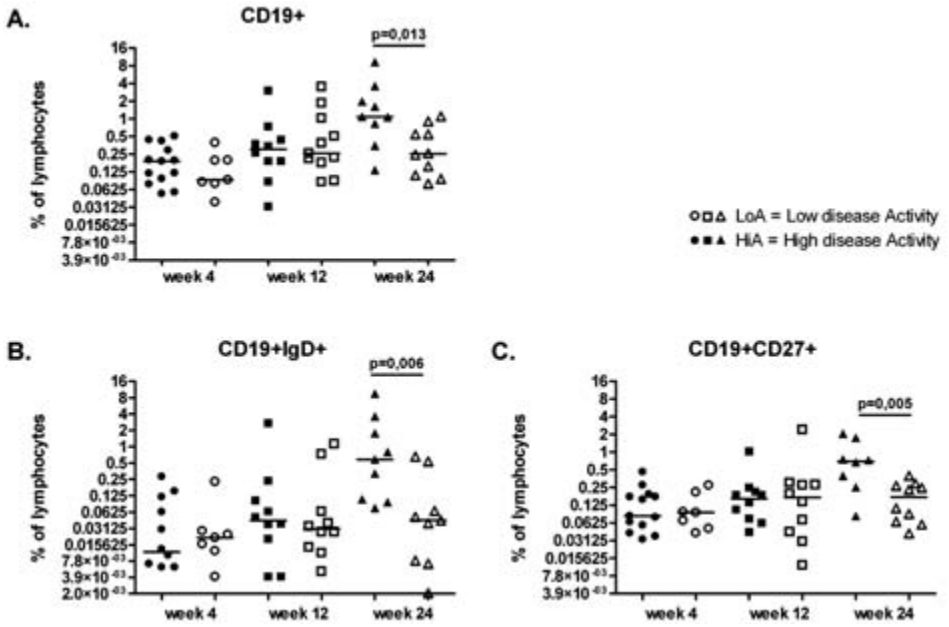


**Figure 3** Repopulation of B-cells after rituximab treatment in patients who achieved low disease activity (LoA-group) and patients who did not (HiA-group). **A:** Percentages of repopulating CD19+ B-cells, **B:** CD19+ IgD+ pre-switched B-cells and **C:** CD19+ CD27+ post-switched B-cells after rituximab treatment. Open symbols represent patients in the LoA-group, closed symbols represent patients in the HiA-group. A $^{2}$log-scale was used for the y-axis to optimize graphic representation.

strongly associated with disease activity after rituximab treatment. Moreover, the finding that B-cell repopulation was significantly reduced in patients achieving a low disease activity following rituximab treatment (LoA-group), suggested that proliferating, and thus differentiating[16,17], B-cells into plasma cells were actively involved in the disease process. Of note, we found similar but less significant results for the association between CD79a+ cells and $DAS_{44}$ area under the curve, as a measure of overall inflammation. Previously, no correlations were reported between changes in disease activity and (changes in) proportions of B-cells in blood, bone marrow or synovium[9] nor did we observe a significant association at the 12 weeks timepoint between B-cells in blood bone marrow or synovium.

Previous studies have shown that CD79a is expressed in B-cell lineage from pre-B-cell stage up to the plasma cell stage[18,19] in contrast to CD138 which is

only expressed on terminally differentiated plasma cells. Also, we previously found a strong and significant correlation between ACPA-IgM serum levels and CD79a+ synovial expression[9]. So, even though double stainings with CD79a and CD138 were lacking in this study, it is conceivable that CD79a+ CD138- plasma cells are a distinct subset of plasma cells, as previously observed in multiple myeloma patients [20;21]. This could explain why CD79a+ and CD138+ plasma cells behaved differently after rituximab treatment: a T-cell dependent reduction of CD138+ plasma cells was observed, which was not directly related to disease activity. Most likely, the latter is best explained by a contact-dependent interaction between T-cells and the survival of long-lived plasma cells in humans, which was recently described[22]. Taken together, these findings indicate that that the synovial load of CD79a+ plasma cells plays a more pivotal role in disease activity in RA patients before and after treatment than CD138+ plasma cells.

The present study demonstrated that effective interference with B-cell proliferation and autoreactive plasma cell formation in synovium was the most likely mechanism through which rituximab reduced disease activity in RA. However, due to the observational design, this study could not completely exclude other possible mechanisms. The LoA-group already had significantly lower $DAS_{44}$ scores at baseline introducing a possible bias. However, a spin-off study of the REFLEX study, a large double-blind, randomized prospective study, already reported that the outcome of retreatment with rituximab improved if disease activity was not allowed to worsen before retreatment[23]. It was found that for every 1.0 point that the $DAS_{28}$ was allowed to worsen resulted in a $0.32 \pm 0.04$ higher $DAS_{28}$ score after a subsequent course of rituximab. These data confirmed that treatment with rituximab was more effective when disease relapse was not full-blown and rituximab more effectively interfered with the pathogenic mechanisms underpinning disease relapse. Intriguingly, it was previously demonstrated that synovium of patients with advanced RA contained more pronounced plasma cell infiltration than of patients with early disease[24]. Therefore, it is probable that the low DAS score in our study was not a bias but part of the plasma cell-associated pathology found in our RA patients. Also, this study did not address the reduction of other cell types by rituximab treatment, in line with a previous report suggesting that B-cells orchestrate synovial cellular infiltration[10]. However, the effects of rituximab on non-B-cell infiltrates cannot explain

the counterintuitive finding that less B-cell infiltration was associated with a better response. If anything, the contrary would be expected. Last but not least, the possibility remained that rituximab was unable to reduce disease activity in the HiA-group due to residual B-cells located at protective sites, such as the peritoneal cavity[25]. Although our early repopulation data supported the hypothesis of residual, post-switched B-cells, these 'resistant' B-cells could not explain the increased reconstitution of pre-switched B-cells. Therefore, the effective interference of rituximab with B-cell proliferation and differentiation towards plasma cells was the most likely mechanism of action explaining the comprehensive observational data of peripheral blood, bone marrow and synovium of rituximab-treated patients.

A limitiation of our study was the extent of B-cell subtyping, which did not allow identification of IgD+CD27+ unswitched memory B-cells. This subset was estimated to comprise 5-15% of the total B-cell population[26]. However their relevance to autoimmune diseases remains unclear yet. Also, the use of corticosteroids in our treatment protocol might have influenced the effects of rituximab on different cell populations including plasma cells[27]. However, in the present study both groups were treated similarly and therefore comparability was preserved. Lastly, the clinical relevance of the present study suggesting that rituximab is most effective in patients who did not yet have high proportions of CD79a+ plasma cells infiltrating inflamed synovium remains speculative. It was previously shown that rituximab did not directly deplete tissue-derived CD79a+ cells[28]. Therefore, it is tempting to speculate that rituximab will be most effective in early RA rather than in longstanding RA, however, it is clear that replication of these data is needed in other cohorts.

In conclusion, the present study demonstrated that the effective reduction of disease activity by rituximab in RA patients could be explained by the presence of CD79a+ plasma cells in synovium and that low disease activity associated with fewer CD79a+ plasma cells and a reduction of B-cell proliferation. Future studies will need to confirm whether B-cell proliferation and plasma cell formation have a pivotal role in RA pathogenesis.

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