Targeting the humoral immune system of patients with rheumatoid arthritis
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Chapter 3

Immunohistochemical analysis as a means to predict responsiveness to rituximab treatment

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Abstract

Objective
Anti-CD20-mediated B-cell depletion with rituximab is a new and effective therapy for rheumatoid arthritis (RA). Although B-cells in peripheral blood (PB) are consistently depleted in all patients, the clinical effects are more heterogeneous, possibly related to differences in the depleting effects of lymphoid or solid tissues. The aim of this study was to investigate B-cell depletion in different compartments (PB, bone marrow, and synovium) and determine predictive variables for responsiveness to rituximab therapy.

Methods
Before and 12 weeks after rituximab treatment, samples of PB, bone marrow, and synovium were collected from 25 patients with RA refractory to disease-modifying antirheumatic drugs and tumor necrosis factor-blocking agents. CD19+ and CD20+ B-cells in PB and bone marrow were measured by flow-cytometric analysis, whereas CD79a+ and cytoplasmic CD20+ B-cells in the synovium were stained by immunohistochemistry. The effects of rituximab on serum Ig and autoantibodies were measured by enzyme-linked immunosorbent assay.

Results
Rituximab effectively depleted the CD20+ subset of B-cells in the PB, bone marrow, and synovium of RA patients. Rituximab significantly reduced autoantibody production (anti-citrullinated protein antibodies [ACPAs] and rheumatoid factor [RF]), in part due to a nonspecific decrease in total Ig production. Importantly, positivity for circulating ACPA-IgM, in combination with a high infiltration of CD79a+ B-cells in the synovium, but not of CD138+ plasma cells, was a predictor of clinical outcome after rituximab treatment. ACPA-IgM titers were independently associated with synovial infiltration of CD20-, CD79a+ B-cells, but not with CD138+ plasma cells.

Conclusion
These data provide novel insights into the mechanisms of CD20-mediated B-cell depletion in the lymphoid and solid tissues of RA patients and suggest a pivotal role for ACPA-IgM producing plasmablasts in RA.

Introduction

B-cells have been implicated in the pathogenesis of rheumatoid arthritis (RA) since the discovery of circulating rheumatoid factor (RF) autoantibodies in RA patients[1]. Recently, anti-citrullinated protein antibodies (ACPAs) have been shown to be more specific for RA[2]. The presence of RF and ACPA autoantibodies is associated with disease progression[3] and responsiveness to treatment with antirheumatic drugs[4,5]. Also, animal studies have shown that B-cells are necessary for the development of arthritis and influence its severity[6]. The successful introduction of B-cell depleting monoclonal antibodies as a treatment for patients with refractory RA confirmed the important role of B-cells in RA. Rituximab is a chimeric monoclonal antibody directed against the B-cell-specific membrane protein CD20[7] that is used in the treatment of B-cell malignancies[8] and was recently approved for use in the treatment of patients with severe, refractory RA[9]. Clinical trials have shown that B-cells are effectively depleted in blood[10,11], but at present, few data exist on tissue effects. The latter might be of importance, since studies have suggested that rituximab is more effective in patients with circulating autoantibodies (ACPA and/or RF) than in patients without these autoantibodies, and this effect was not related to the depletion of circulating B-cells[12].

In order to gain more insight into the mechanistic effects of B-cell depletion in RA, we investigated the effects of anti-CD20-mediated B-cell depletion in peripheral blood (PB), bone marrow, and synovium of patients with severe, refractory RA. In addition, the effects of rituximab on serum concentrations of total Ig (IgM, IgG, and IgA) and autoantibodies were examined. Last, we analyzed which immunologic characteristics in PB, bone marrow, or synovium at baseline predicted responsiveness to rituximab.

Patients & Methods

Patients

The present study involved patients with severe RA who participated in a single-center, open-label trial to investigate the clinical and immunologic effects of treatment with rituximab. All patients had failed treatment with a combina-

tion(s) of disease-modifying antirheumatic drugs (DMARDs) and/or tumor necrosis factor (TNF)-blocking agents. Rituximab was administered as a 1000 mg intravenous infusion on days 1 and 15. As premedication, 100 mg methylprednisolon and 2 mg clemastine were administered intravenously, and 1000 mg acetaminophen was administered orally. TNF-blocking agents were discontinued with a washout period of 8 weeks, whereas DMARDs were continued at the same dosage (methotrexate 2.5-25 mg/week in 21 patients, prednisolone 5-20 mg/day in 10 patients, and leflunomide 20 mg/day in 1 patient). The followup in the current study was 24 weeks. The study was approved by the Ethics Committee of the Leiden University Medical Center (LUMC), and all patients provided written informed consent.

Clinical efficacy

Clinical efficacy was assessed using the Disease Activity Score (DAS) for RA (13), performed by one research nurse. The DAS is a validated combined index used to measure the disease activity in RA patients. Three or 4 variables are assessed, as follows: number of swollen and tender joints (4 joints assessed), erythrocyte sedimentation rate or C-reactive protein level, and global health measured on a 100 mm visual analog scale. Clinical responses were categorized according to the European League Against Rheumatism (EULAR) response criteria[13].

Sample collection

Heparinized blood samples and bone marrow aspirates were obtained jointly at baseline and 12 weeks after the initiation of B-cell depleting therapy. Peripheral blood mononuclear cells (PBMCs) and bone marrow mononuclear cells (BMMCs) were isolated by density-gradient centrifugation over Ficoll-amidotrizoaat (LUMC, Leiden, The Netherlands). Serum samples were collected at baseline and 4, 12, 18 and 24 weeks after the first infusion of rituximab.

Arthroscopy and synovial tissue sampling

Arthroscopy in clinically affected knees of all patients was performed 1-3 weeks before initiation of rituximab treatment, as previously described[14]. The procedure was repeated 12 weeks after the first infusion of rituximab. At the end of each procedure, 80 mg intra-articular prednisolone was administered

only if there was painful swelling of the knee. Biopsies were repeated at 3 months in the same knee (n=17) unless it was previously injected with prednisolone, in which case the biopsy was performed contralateral knee (n=8). On each occasion, 16-20 pieces of synovial tissue were collected using a 2 mm grasping forceps (Storz, Heidelberg, Germany).

Immunohistochemical analysis

Paraffin-embedded consecutive sections were selected for staining with mouse anti-human CD20 (CD20cy) (clone L26) and mouse anti-human CD79a (clone JCD117). The remainder of the serial sections were stained with mouse anti-human Ki-67 (clone MIB-1), mouse anti-human CD68 (clone KP1), rabbit anti-mouse Ig isotype control (code X0936) (all from Dako, Heverlee, Belgium), rabbit anti-human CD3 (clone SP7; Neo-Markers, Fremont, CA), mouse anti-human CD138 (clone B-B4; Serotec, Oxford, UK), mouse IgG1-biotin isotype control (clone J1D9; Ancell/Kordia, Leiden, The Netherlands), and purified mouse IgG1 isotype control (BD PharMingen, San Diego, CA).

Paraffin-embedded sections were deparaffinized using xylol, ethanol, and demineralised water, and then antigens were retrieved by incubating the sections for 10 minutes in 1 mM boiling EDTA or 10 mM citrate buffer. After the sections had been washed in demineralised water and phosphated buffered saline (PBS), the appropriate titrated amount of antibody was added and incubated for 60 minutes at room temperature. After washing with PBS, the sections were incubated with EnVision Mouse conjugate or EnVision Rabbit conjugate (Dako) for 30 minutes. The color reaction was completed with DAB+ substrate (Dako). Counterstaining was performed with hematoxyline. Sections were covered with Micromount mounting medium (Surgipath, Bretton, UK).

Semiquantitative scoring of inflammation

Stained sections were coded and randomly analyzed. All areas of each biopsy section were scored blindly by two independent observers (YKOT and EWNL). Stained sections were scored semi-quantitatively from 0 to 4 (1 = lowest and 4 = highest level of expression). The scoring system was calibrated for each marker separately because some markers are more abundantly expressed than others. Interobserver 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 $P < 0.001$). Differences between the observers were resolved by mutual agreement.

Flowcytometric analysis

Freshly isolated PBMCs and BMMCs were immediately stained for flow cytometric analysis. Staining was performed by incubating fresh mononuclear cells with mouse anti-human monoclonal antibodies in PBS/1.0% bovine serum albumin at 4°C for 30 minutes. The following monoclonal antibodies were used and titrated to determine the optimal concentration: fluorescein isothiocyanate-labeled anti-CD20 (clone 2H7), phycoerythrin-labeled anti-CD19 (clone H1B19), phycoerythrin-labeled anti-CD27 (clone L128), peridinin chlorophyll protein (PerCP)-Cy5.5-labeled anti-CD19 (clone SJ25C1), PerCP-Cy5.5-labeled anti-CD38 (clone HIT-2), and allophycocyanin-labeled anti-CD3 (UCHT-1) (all from Becton Dickinson, San Jose, CA). After incubation, cells were fixed in 4% paraformaldehyde (LUMC) and analyzed within 24-48 hrs. Stained cells were analyzed with a FACSCalibur (Becton Dickinson) flow cytometer, and the associated software program FlowJo (Tree Star, Ashland, OR) was used to calculate frequencies within the lymphocyte population. The detection limit of flow cytometric analysis, below which depletion of CD19+ or CD20+ B-cells was regarded as complete, was set at a frequency of 1% within the total lymphocyte gate.

Measurements of serum antibody titers

Serial serum samples of each patient were analyzed for titers of total Ig, autoantibodies, and exogenous antigen-specific antibodies. Total serum IgG, IgM and IgA titers were measured by immuno-turbidimetric assay using the Cobas Integra 400/700/800 analyzer (Roche Diagnostics, Indianapolis, IN), according to the manufacturer's guidelines.

Serum titers of ACPA of the IgG isotype and of the IgM isotype were measured using a commercial enzyme-linked immunosorbent assay (ELISA) (Immunoscan RA, Mark 2; Euro-Diagnostica, Arnhem, The Netherlands), according to the manufacturer's instructions[15]. Briefly, microtiter plates coated with cyclic citrullinated peptides (Euro-Diagnostica) were incubated for 2 hours with 100 μl/well of serum samples (1:50 dilution). This, and each subsequent incubation step, was performed at 37 °C in a humidified atmosphere and was followed by

washing with washing buffer (Euro-Diagnostica). All samples and reagents were diluted in dilution buffer (Euro-Diagnostica).

To dectect ACPA-IgM, plates were incubated for 2 hours with 100 μl/well of goat anti-human IgM (1:1000 dilution) and conjugated with alkaline phosphatase (AHI 0605 and AHI 0105; BioSource International, Camarillok, CA). The presence of ACPA-specific antibodies was detected using 4-nitrophenyl phosphate disodium salt (Sigma-Aldrich, Steinheim, Germany) as substrate, as previously described[16]. A series of successive dilutions of pooled patient sera[15] were used as a reference standard in all plates. A 1:25 dilution of this standard was defined as containing 1,000 arbitrary units per mililiter (AU/ml) of ACPA-IgM. Serum titers of IgM rheumatoid factor (IgM-RF) were measured using a standardized ELISA, as previously described[17].

Statistical analysis

Results are expressed as the mean ± SEM. Nonparametric Wilcoxo's signed rank tests were used to compare related variables of different time points during followup in the total group of RA patients. Mixed regression analyses were used to compare the slopes of different autoantibody reductions versus stable titers during followup of rituximab treatment. Nonparametric Mann-Withney U tests were used to compare baseline characteristics between good and moderate responders and nonresponders to treatment. Pearson's correlation coefficients and risk estimates were used to calculate odds ratios for achieving a good response according to the EULAR response criteria. Univariate linear regression analyses were used to correlate changes in autoantibody titers with their corresponding Ig concentrations and to correlate immunological variables with ACPA-IgM titers. Multivariate linear regression with a backward method was used to identify independent predictive variables for ACPA-IgM. P values less than or equal to ≤ 0.05 were considered significant, and a trend toward significance was defined as $P < 0.10$.

Results

Study patients and sampling

Twenty-five patients with severe, refractory RA were included in the study. The patients' characteristics are shown in Table 1. Briefly, 18 patients (72%) were female, 24 (96%) were positive for IgM-RF, 22 (88%) were positive for ACPA-IgG, 24 (96%) had erosive disease, and all patients had failed to respond to treatment with multiple DMARDs and TNF-blocking agents. Significant reductions in DAS scores were observed after rituximab treatment (median at baseline: 3.60 [range 2.17-5.23], at 12 weeks 2.42 [range 1.09-3.41]; ($P<0.001$), with 8 patients (32%) achieving a good response, 13 patients (52%) achieving a moderate response, and 4 patients (16%) achieving no response, according to the EULAR response criteria. Paired sampling of PBMCs, BMMCs, and synovial biopsies was complete in 17 of 25 patients; PBMCs were collected from 24 patients (1 patient had too few circulating leucocytes), BMMCs were collected from 18 patients (3 patients refused bone marrow aspirations, 2 patients failed the procedure, and 2 patients had no followup aspiration), and synovial biopsies were performed in 17 patients (2 patients refused arthroscopy, 1 patient was taken anticoagulants, and 5 patients did not undergo followup arthroscopy). Synovial biopsies were performed at a median of 13 weeks after initiation of rituximab treatment (range 9-21 weeks).

Depletion of CD20+ B-cells from PB, bone marrow, and synovium after rituximab treatment

To establish whether rituximab was able to deplete B-cells in different compartments, the percentages of CD19+ and CD20+ B-cells in PB and bone marrow and of CD79+ and cytoplasmic CD20+ B-cells in the synovium was determined. B-cells were mainly CD19+ and CD20+ in PB and bone marrow (Figures 1A and B). In blood, absolute numbers of B-cells before treatment ranged from 36 to 718 $x10^6$ cells/L, corresponding to 1.77-18.4% of total lymphocytes. In the synovium, cell populations positive for CD20cy and CD79a could be identified (Figure 1C). In PB, depletion of CD20+ B-cells was complete in 24 of 25 patients (96%) and depletion of CD19+ B-cells was complete at 12 weeks in 22 of 25 patients (88%) (Figure 1A). Importantly, at 4 weeks after rituximab treatment, the blood of all 25 patients was completely depleted of CD20+ as well as CD19+ B-cells. In bone marrow, depletion of CD20+ B-cells was com-

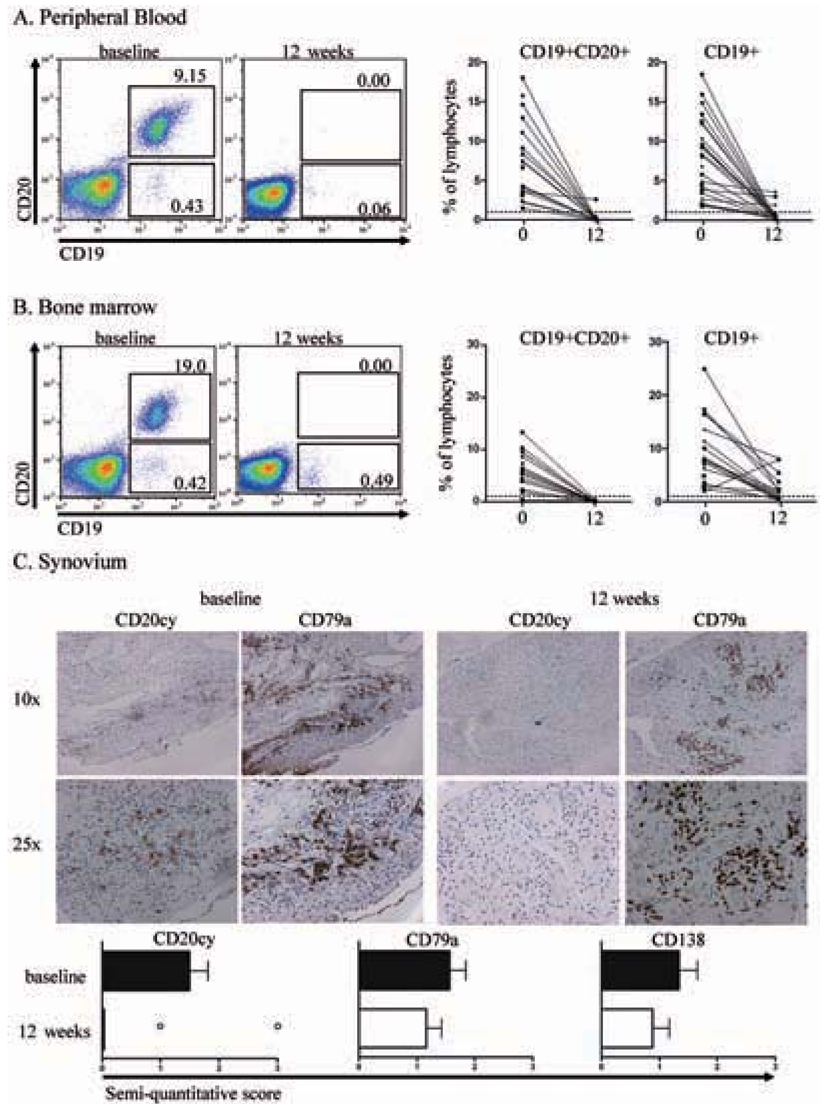

Figure 1 B-cells in peripheral blood, bone marrow, and synovium before and 12 weeks after rituximab treatment. **A:** CD20 and CD19 expression on CD3- lymphocytes in peripheral blood from a rheumatoid arthritis (RA) patient (left). Numbers represent the percentages of total lymphocytes. Flow cytometric data on 24 individual RA patients are shown on the right. The broken line represents the detection limit. **B:** CD20 and CD19 expression on CD3- lymphocytes in bone marrow from the same RA patient (left). Flow cytometric data on 18 individual RA patients are shown on the right. **C:** Consecutive sections of synovium stained for cytoplasmic CD20 (CD20cy) and CD79a (top). Semi-quantitative scoring of CD20cy, CD79a, and CD138 expression (0=no expression; 4=highest level of expression) in synovial specimens from 17 RA patients is shown at bottom. Values are the mean and SEM. Circles indicate outliers.

plete in all patients; however, depletion of CD19+ B-cells was complete in 8 of 25 patients (32%), with a residual median percentage of CD19+ B-cells of 2.21% [range 1.05-8.11] (Figure 1B). Last, in the synovium (Figure 1C), depletion of cytoplasmic CD20+ B-cells was complete in 15 of 17 patients (88%), whereas CD79a+ B-cells and CD138+ plasma cells were not depleted in any of the patients.

We found no differences in clinical response to rituximab when comparing patients with incomplete versus complete depletion of either CD19+ B-cells in bone marrow or of CD79a+ B-cells in synovium. Of note, after rituximab treatment, no significant differences were found in percentages of CD3+, CD4+ and CD3+, CD8+ T-cells, CD16+, CD56+ natural killer cells, or CD14+ monocytes in blood or bone marrow. In the synovium, there was no significant change in CD3 expression, although a trend toward a decrease ($P < 0.10$) was observed of CD68 and Ki-67 expression (data not shown).

Table 1 Characteristics of the 25 patients

Age, median (range) years	55 (34-82)
Female, (%)	72
White, (%)	92
DAS at baseline, median (range) score	3.60 (2.17-5.23)
Disease duration, median (range) years	14 (3-53)
RF positive, %	96
ACPA IgG positive, %	88
ACPA IgM positivity, (%)	50
Erosive disease, %	96
No. of DMARDs used without success, median (range)	5.4 (2-11)
No. of TNF-blocking agents used without success, median (range)	1.4 (0-3)
Lowest DAS, median (range) score	2.42 (1.09-3.71)†
EULAR response, %	
Good	32
Moderate	52
None	16

*DAS=Disease Activity Score; RF=rheumatoid factor; ACPA=anti-citrullinated protein antibody; DMARDs=disease modifying antirheumatic drugs; TNF=tumor necrosis factor; EULAR=European League Against Rheumatism. † $P < 0.001$ versus baseline DAS score, by nonparametric Wilcoxon's signed rank test

Reductions in ACPA-IgG, ACPA-IgM, and IgM-RF autoantibody production after rituximab treatment

Rituximab treatment led to significant reductions in serum total IgG (median [range] baseline 12.0 gm/liter [5.50-20.7] versus nadir 10.5 [5.40-15.1]) (*P*=0.001), total IgM (baseline 1.30 gm/liter [0.60-3.60] versus nadir 1.0 [0.30-2.10] (*P*<0.001), and total IgA (baseline 1.95 gm/liter [1.0-6.35] versus nadir 1.60 [1.0-5.70] (*P*=0.001). Also, ACPA IgG titers decreased significantly from 443 AU/ml (range 56-1,280] at baseline to a nadir of 364 AU/ml (44-897) (*P*=0.012), IgM-RF titers decreased from 49 IU/ml (14-205) at baseline to a nadir of 25 (1-184) (*P*<0.001), and ACPA IgM titers decreased from 61 AU/ml (34-251) at baseline to a nadir of 34 (20-71) (*P*=0.009) (Figure 2A). Of note, in the majority of patients a gradual decrease was observed, generally reaching lowest levels at 24 weeks after rituximab treatment.

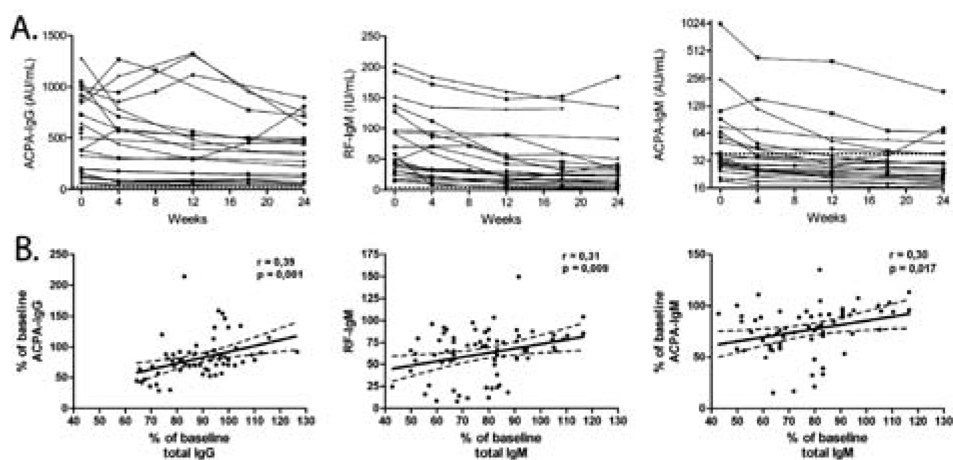

Figure 2 Effects of rituximab treatment on Ig titers. **A:** Titers of anti-citrullinated protein antibody (ACPA)-IgG, IgM rheumatoid factor (IgM-RF) and ACPA-IgM during a 24-week period in rheumatoid arthritis patients treated with rituximab. Broken lines represent the validated cutoff values for positivity. Due to a large spread of the ACPA-IgM titers, a ^{2}log-transformed y-axis was used. **B:** Nonspecific reductions of autoantibody titers after rituximab treatment, as determined by calculating the correlations between the percentage change in titers of ACPA-IgG and total IgG levels, percentage change in titers of IgM-RF and total IgM levels, and percentage change in titers of ACPA-IgM and total IgM levels. Broken lines represent the 95% confidence interval of the linear regression (solid lines).

Interestingly, the percentage change in titers of ACPA-IgG versus total IgG (r=0.39, *P*=0.001), IgM-RF versus total IgM (r=0.31, *P*=0.009), and ACPA-IgM versus total IgM (r=0.30, *P*=0.017) showed significant correlations (Figure 2B). However, at 24 weeks, a significantly lower percentage of circulating autoantibodies than serum Ig remained after rituximab treatment, comparing ACPA IgG with total IgG (median [range] 72% [28-213] versus 84% [66-127]; *P*=0.035) and IgM-RF with total IgM (54% [8-96%] versus 67% [43-91%]; *P*=0.011). However, the percentage change in ACPA-IgM from baseline was not significantly lower than that of total IgM (72% [15-111%] versus 67% [43-91%]; *P*=0.50). These data indicated that the depletion of CD20+ B-cells by rituximab induced a non-specific reduction in circulating autoantibodies, notably, ACPA-IgM, but also ACPA-IgG and IgM-RF.

Variables associated with good response to rituximab treatment

We investigated whether clinical and immunological variables in PB, bone marrow, or synovium were associated with a clinical response to rituximab treatment by stratifying RA patients according to the EULAR response criteria[13]. By comparing patients with a good response (n=8) with patients with a moderate or no response (n=18), we found that, at baseline, good responders had a significantly lower infiltration of CD20+ B-cells (mean ± SEM 0.29 ± 0.28 versus 2.14 ± 0.38 in those with moderate or no response) (*P*=0.005) as well as CD79a+ B-cells (0.62 ± 0.29 versus 2.05 ± 0.34 in those with moderate or no response) (*P*=0.01) (Figure 3A). In addition, expression of CD138 was significantly lower in good responders (0.43 ± 0.30) than in patients with moderate or no response (1.79 ± 0.42) (*P*=0.046), which was also reflected in the number of CD138+ cells (1.97 ± 1.84 versus 43.6 ± 20.7 in those with moderate or no response) (*P*=0.016). Furthermore, good responders had less synovial inflammation, which was supported by significantly lower Ki-67 expression (0.33 ± 0.21 versus 2.07 ± 0.43 in those with moderate or no response) (*P*=0.032) and a trend toward lower overall inflammation scores (7.42 ± 1.84 versus 13.5 ± 1.73 in those with moderate or no response) (*P*=0.07) and CD3+ T-cell scores (1.50 ± 0.56 versus 2.86 ± 0.33 in those with moderate or no response) (*P*=0.06) (Figure 3A).

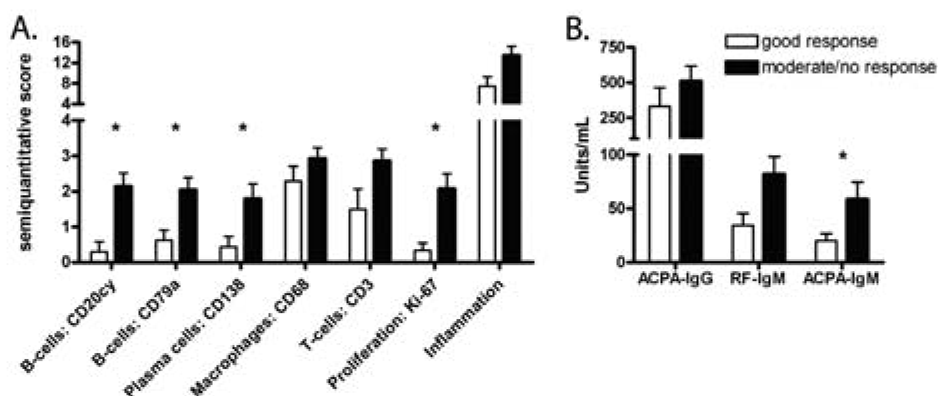

Figure 3 Differences in baseline characteristics of good responders versus those with moderate or no response, as classified according to the European League Against Rheumatism response criteria. **A:** Expression of specific markers for B-cells (cytoplasmic CD20 [CD20cy], CD79a), plasma cells (CD138), macrophages (CD68), T cells (CD3), proliferation (Ki-67), plasma cell counts, and total inflammation. **B:** Circulating titers of rheumatoid arthritis-specific autoantibodies ACPA-IgG, IgM-RF, and ACPA-IgM. Values are the mean and SEM. *=$P \leq 0.05$; #=$P<0.10$. NS= not significant (see Figure 2 for other definitions).

Additionally, patients with a good response to rituximab treatment had significantly lower ACPA-IgM titers at baseline (20 ± 6.6 units/ml) compared with those with moderate or no response (59 ± 15.8 units/ml) (*P*=0.038), as well as lower IgM-RF titers (34 ± 11.3 versus 82 ± 16 units/ml in those with moderate or no response) (*P*=0.075) (Figure 3B). Although ACPA-IgG titers were lower in good responders, there was no significant difference at baseline (330 ± 134 versus 512 ± 105 units/ml in those with moderate or no response) (*P*=0.23). Importantly, there were no differences in total serum IgG, IgM and IgA between the groups. Also, no differences were found for good responders compared with those with moderate or no response with respect to clinical characteristics (age, sex, disease duration, DAS), percentages of CD20+ or CD19+ B-cells in blood or bone marrow, and absolute numbers of B-cells in blood (data not shown). Also, we found no biologic or inflammatory marker that was significantly associated with improvement of clinical disease activity.

Serum ACPA-IgM titer in combination with high CD79a or Ki-67 expression in synovium, but not with CD138 expression, predicts outcome after rituximab treatment

The striking differences in synovium infiltration and serum levels of autoantibodies between good responders and those with moderate or no response led us to identify variables that could predict clinical outcome of rituximab therapy. Therefore, we dichotomized groups for ACPA-IgM, using the median titer of 33 AU/ml as a cutoff; for synovial B-cell expression, using the CD79a expression median score of 1 as a cutoff; and for plasma cell expression, using the CD138 expression median score of 1 as a cutoff. Table 2 shows that patients with a negative ACPA-IgM status had a significantly lower risk of achieving a moderate or no response to rituximab treatment (odds ratio [OR] 0.56, 95% confidence interval [95% CI] 0.32-1.0). Also, patients with low expression of CD79a (OR 0.51, 95% CI 0.26-1.0) or Ki-67 (OR 0-46, 95% CI 0.24-0.87) in synovium had a significantly lower risk for a moderate or no response. This was not the case for CD138 expression (OR 0.62, 95% CI 0.35-1.09). When each score for synovial markers was combined with serum ACPA-IgM status, the risk of a moderate or no response to rituximab was significantly lower in patients with a negative ACPA IgM status and low expression of CD79a (OR 0.38, 95% CI 0.15-092) or Ki-67 (OR 0.29 95% CI 0.09-0.92) in synovium (Table 2).

Correlation of serum ACPA-IgM titers with CD79a expression in synovium before and after depletion of CD20+ B-cells

In the prediction analysis, ACPA-IgM appeared to be closely related to B-cell lineage markers in synovium, suggesting that ACPA-IgM production is possibly derived from activated B-cells in synovium. To further corroborate which subset of the synovial B-cell population was associated with ACPA-IgM titers, we investigated which variables in synovium were related to serum ACPA-IgM titers before and after CD20+ B-cells depletion (Table 3).

We showed that before B-cell depletion, ACPA-IgM strongly correlated with DAS scores (r=0.53, *P*=0.008) and correlated well with CD79a (r=0.42, *P*=0.067), Ki-67 (r=0.45, *P*=0.054), and CD138 expression (r=0.49, *P*=0.028). After the depletion of CD20+ B-cells, ACPA-IgM correlated strongly with CD79a expression in synovium (r=0.69, *P*=0.003) and moderately with Ki-67

Table 2 Odds ratios of achieving moderate or no response after rituximab treatment*

	No. of patients/ total	OR (95% CI)
Individual variable (score)		
ACPA-IgM status†		
Negative (0)	7 / 14	0.56 (0.32-1.0) ‡
Positive (1)	8 / 9	1.0 (referent)
CD79a expression§		
Low (0)	5 / 11	0.51 (0.26-1.0) ‡
High (1)	9 / 10	1.0 (referent)
Ki-67 expression§		
Low (0)	5 / 11	0.46 (0.24-0.87)‡
High (1)	9 / 9	1.0 (referent)
CD138 expression§		
Low (0)	7 / 13	0.62 (0.35 – 1.09)
High (1)	7 / 8	1.0 (referent)
Combined synovial and serum ACPA IgM status (score)		
CD79a expression		
Low (0)	3 / 8	0.38 (0.15-0.92) ‡
Intermediate (1)	4 / 6	0.67 (0.38-1.17)
High (2)	5 / 5	1.0 (referent)
Ki-67 expression		
Low (0)	2 / 7	0.29 (0.09-0.92) ‡
Intermediate (1)	5 / 6	0.83 (0.58-1.19)
High (2)	6 / 6	1.0 (referent)
CD138 expression		
Low (0)	4 / 10	0.48 (0.21-1.11)
Intermediate (1)	3 / 3	1.20 (0.84-1.72)
High (2)	5 / 6	1.0 (referent)

*Odds ratios (ORs) of achieving a good response could not be calculated because there were not enough patients (n < 5) when groups were dichotomized or trichotomized for each variable. In a first analysis, patients received a score of 0 or 1 for the individual variables measured in serum or synovial biopsy specimen; in a second analysis, patients' scores for serum anti-citrullinated protein antibody (ACPA) IgM were combined with their corresponding score for synovial markers. 95% CI = 95% confidence interval.
† Positivity was defined as titers greater than the overall median of 33 arbitrary units/ml.
‡ $P \leq 0.05$ versus referent group, by Pearson's correlation coefficient.
§ High expression was defined as values greater than the overall median of the semiquantitative scores.

expression (r=0.47, *P*=0.056), but not with CD138 expression (r=-0.19, *P*=0.462). Importantly, after depletion, ACPA-IgM correlated well with total serum IgM (r=0.43, *P*=0.038), which is predominantly derived from residual CD20-, IgM-producing plasmablasts. In a multivariate regression analysis, CD79a expression was shown to be independently associated with ACPA-IgM titer after CD20+ B-cell depletion (β=0.657, *P*=0.003). IgM-RF titers did not correlate significantly with any of the above-mentioned variables.

Table 3 Correlation of ACPA-IgM levels with clinical disease activity and with CD79a+ B-cells in synovium, before and after rituximab-mediated depletion of CD20+ B-cells*

	r	***P***
Before rituximab		
DAS score	0.53	0.008
Total IgM levels	0.18	NS
Synovial infiltration		
CD79a	0.42	0.067
Ki-67	0.45	0.054
CD138	0.49	0.028
CD20	0.16	NS
After rituximab		
DAS score	0.095	NS
Total IgM levels	0.43	0.038
Synovial infiltration		
CD79a	0.69	0.003
Ki-67	0.47	0.056
CD138	-0.19	NS
CD20	-0.11	NS

*r values > 0.50 were considered strong correlations, r values < 0.29 weak correlations. NS = not significant (see Table 1 for other definitions) [43]

Discussion

The aim of the study was to investigate the depleting effects of rituximab in the PB, bone marrow, and synovium of patients with refractory RA and to identify predictive variables for responsiveness to rituximab treatment. Our study showed that depletion of CD20+ B-cells is complete in all 3 compartments in the majority of patients. However, not all B lineage cells were completely depleted, as illustrated by the persistence of CD19+ B-cells in bone marrow and CD79a+ B-cells in synovium after rituximab treatment. B-cell depletion led to significant reductions in serum Ig, which was also reflected in significantly lower titers of ACPA-IgG, ACPA-IgM and IgM-RF autoantibodies. Additionally, we showed that high ACPA-IgM status, combined with high synovial B-cell infiltration, was predictive of moderate or no response to rituximab treatment. Moreover, we were able to show that ACPA-IgM and synovial B-cell infiltration were closely linked in RA patients, irrespective of the presence of CD20+ B-cells, suggesting an important role of ACPA-IgM-producing plasmablasts in the persistence of RA.

To our knowledge, this is the first comprehensive study to examine the effects of B-cell depletion by rituximab in 3 different compartments, i.e., PB, bone marrow, and synovium. Previous studies in RA patients have focused mainly on PB as a measure of effective depletion of B-cells[10,18,19]. Although in one animal study, it was shown that depletion of B-cells in bone marrow and lymph nodes is significant but incomplete[20], studies analyzing the degree of depletion of B-cells in lymphoid tissues in humans are limited. Leandro *et al.*[21] reported incomplete depletion of CD19+ B-cells in bone marrow of 6 RA patients 3 months after rituximab treatment. They found very low percentages of CD20+ B-cells in bone marrow after rituximab treatment, which is consistent with our findings, but baseline samples were not studied.

Recently, the depleting effects of rituximab on synovial B-cells from RA patients at 4 weeks after treatment were reported[22]. That study showed a significant decrease in, but incomplete depletion of, CD22+ B-cells in synovium. Because CD22 and CD20 are not jointly expressed during the differentiation of immature B-cells into mature B-cells and eventually into plasma cells, it is difficult to compare the results reported by Vos *et al.*[23-27] with those of our study.

CD20 is expressed earlier in the development of B-cells than is CD22. Circulating IgM+, IgD+ naive B-cells express both CD20 and CD22[23-27], and CD22 expression is closely linked to surface Ig expression, notably surface IgD, on B-cells. Importantly, germinal center B-cells, plasma cells, and in vitro-activated B-cells cease to express CD22[28,29]. The use of methylprednisolon in our study and the earlier time point of analysis (4 weeks after initiation of rituximab treatment) used by Vos *et al.* might explain the different findings.

Taken together, these studies clearly indicate that the percentage of CD19+ B-cells in PB is a suboptimal reflection of the B-cell compartment in RA patients treated with rituximab. However, our study indicates that a single treatment with 2 infusions of 1,000 mg of rituximab is able to completely deplete the subset of CD20+ B-cells in PB as well as bone marrow and synovium in RA patients.

Consistent with a previous study by Cambridge *et al.*[30], we found that the significant reductions in ACPA-IgG, IgM-RF and ACPA-IgM titers partly reflected a nonspecific reduction of total Ig concentrations. This phenomenon was probably due to the elimination of early, CD20-expressing plasmablasts by rituximab, which could also explain the more profound reduction of total IgM titers relative to IgG and IgA. However, we cannot rule out the possibility that a reduction of inflammatory signals was simultaneously responsible for the reduced concentrations of Ig, including autoantibody titers, after rituximab treatment. In a large randomized study of 160 patients, comparable reductions of total Ig, but not of IgG antibodies against tetanus toxoid (TT), were reported[10]. Although it was unclear whether TT-IgG titers were high enough to detect any effect of B-cell depleting therapy, that study did suggest that rituximab has little influence on existing antibody titers against exogenous antigens when they are predominantly derived from terminally differentiated plasma cells.

The current study showed that after rituximab therapy, residual CD19+, CD20- B-cells could be found in bone marrow, presumably, these were stem cells, early pro-B-cells, and terminally differentiated plasma cells[31]. This also seemed to apply to the synovium, where we found no change in the infiltration of CD138+ plasma cells. Also, we showed that rituximab did not lead to significant changes in other lymphocyte subsets in blood and bone marrow, and only a

trend toward significance was found for the reduction of CD68+ macrophage infiltration and Ki-67+ proliferating cells in the synovium. Recently, it was shown in a SCID mouse model that tonsillar plasma cell survival depended upon contact-dependent interactions with T-cells[32]. Therefore, it is conceivable that the residual lymphocyte subsets in the synovium, including T-cells and plasma cells, might be responsible for the maintenance of an inflammatory environment, and as a result, reemerging and reinfiltrating B-cells could trigger a relapse of synovitis. The latter could explain the temporary beneficial effects of B-cell depleting therapy in RA patients, although it remains unclear why some patients achieved long-term ameliorations despite the return of circulating B-cells.
Our study is the first to identify predictive factors of responsiveness to rituximab, namely, low baseline infiltration of B-cells in the synovium together with low titers of ACPA-IgM. Previously, it was reported that RA patients negative for both ACPA-IgG and IgM-RF responded worse to rituximab treatment than RA patients who where positive for both ACPA-IgG and IgM-RF[12]. Even though we could not confirm this finding because our study included only 1 patient who was negative for both ACPA-IgG and IgM-RF, we did find a strikingly higher concentration of IgM autoantibodies (ACPA-IgM and IgM-RF) in patients who showed only a moderate or no response to rituximab.

Moreover, we showed that the concentration of ACPA-IgM, was closely related to CD79a+ B-cell expression in synovium. Because this relationship was preserved in patients before and after rituximab treatment, the ACPA-IgM titer was independent of the presence of CD20+ B-cells. Importantly, ACPA-IgM was also not related to CD138 expression in the synovium, indicating that ACPA-IgM correlated with CD79a+ B-cells, irrespective of CD20 or CD138 expression. Previous studies have shown that ACPA can be produced in synovial tissue[33] and the presence of different isotypes of ACPA has been shown in RA patients[15,34]. However, the production of ACPA-IgM in the synovium has not yet been studied. Therefore, it remains unclear whether ACPA-IgM is indeed derived from synovial plasmablasts, although several studies have already demonstrated that B-cells clonally expand[35,36] and locally differentiate[37,38] in chronically inflamed synovium. It is therefore tempting to speculate that patients with a high level of synovial infiltration by autoantibody-producing

plasma cells might benefit from retreatment with rituximab in order to indirectly eliminate autoreactive, CD20- plasma cells.

Previous studies[39,40] have demonstrated the possible internalization of the CD20 protein after rituximab treatment, and it has also been suggested that rituximab antibody can mask the epitope, resulting in false-negative expression of CD20. The current study was designed to overcome the issue of possible masking of the CD20 membrane protein by rituximab through the simultaneous staining of CD19 and CD20 by flow cytometry and the staining of the cytoplasmic tail of the CD20 protein in synovial sections. Because the reduction of CD19+ and CD20+ B-cells correlated well in the overall group as well as in consecutive samples of individual patients, we believe epitope masking did not influence our results on depletion in blood and bone marrow. The possible internalization of the CD20 protein could not be excluded in our immunohistochemical analysis. However, internalization of CD20 was shown in vitro to be a temporary phenomenon (maximum 48 hours) and was therefore less likely to have influenced the stainings of synovial specimens obtained 3 months after initiation of rituximab treatment. Moreover, with the goat anti-mouse isotype control, which is able to bind mouse epitopes of rituximab[39], results were negative (data not shown).

As mentioned above, a limitation of our study is the possible influence of methylprednisolone, which was administered with each rituximab infusion and has been shown to influence plasma cell survival[41]. Intravenous methylprednisolone could have affected the measured Ig levels. Also, in 3 patients we observed early reconstitution of low percentages of B-cells in PB at 12 weeks after rituximab treatment, which might have influenced the measured B-cell depletion in bone marrow and synovium from these patients at that time. Of note, in these 3 patients, we could discern neither a distinct clinical phenotype nor a lack of clinical response to rituximab. The majority of patients (88%) had complete depletion of circulating B-cells at the time bone marrow and synovium were collected.

In conclusion, our study showed complete depletion of the CD20+ subset of B-cells in PB, bone marrow, and synovium of RA patients, resulting in a significant but partly nonspecific reduction of autoantibodies. Furthermore, ACPA-

IgM status, together with synovial infiltration of B-cells at baseline, was predictive of clinical outcome after rituximab treatment. It was further shown that ACPA-IgM titers were closely related to synovial B-cell infiltration, independently of the presence of CD20+ B-cells. Collectively, the findings of this study provide novel insights into the effects of B-cell depletion in RA and the role of synovial B-cells in RA-specific autoantibody production.

References

1. Rose HM *et al*. Differential agglutination of normal and sensitized sheep erythrocytes by sera ofpatients with rheumatoid arthritis. *Proc Soc Exo Biol Med* **68**: 1-6 (1948)
2. Avouac J *et al*. Diagnostic and predictive value of anti-cyclic citrullinated protein antibodies in rheumatoid arthritis: a systematic literature review. *Ann Rheum Dis* **65**: 845-51 (2006)
3. Berglin E *et al.* Radiological outcome in rheumatoid arthritis is predicted by presence of antibodies against cyclic citrullinated peptide before and at disease onset, and by IgA-RF at disease onset. *Ann Rheum Dis* **65**: 453-8 (2006)
4. Braun-Moscovici Y *et al.* Anti-cyclic citrullinated protein antibodies as a predictor of response to anti-tumor necrosis factor-alpha therapy in patients with rheumatoid arthritis. *J Rheumatol* **33**: 497-500 (2006)
5. Bobbio-Pallavicini F *et al.* High IgA rheumatoid factor levels are associated with poor clinical response to tumour necrosis factor {alpha} inhibitors in rheumatoid arthritis. *Ann Rheum Dis* **66**: 302-7 (2007)
6. Dunussi-Joannopoulos K *et al.* B-cell depletion inhibits arthritis in a collagen-induced arthritis (CIA) model, but does not adversely affect humoral responses in a respiratory syncytial virus (RSV) vaccination model. *Blood* **106**: 2235-43 (2005)
7. Silverman GJ. Therapeutic B-cell depletion and regeneration in rheumatoid arthritis: emerging patterns and paradigms. *Arthritis Rheum* **54**: 2356-67 (2006)
8. McLaughlin P. Rituximab: perspective on single agent experience, and future directions in combination trials. *Crit Rev Oncol Hematol* **40**: 3-16 (2001)
9. Mabthera. Summary of product characteristics. 2006. Ref Type: Internet Communication
10. Edwards JC *et al.* Efficacy of B-cell-targeted therapy with rituximab in patients with rheumatoid arthritis. *N Engl J Med* **350**: 2572-81 (2004)
11. Leandro MJ *et al*. Reconstitution of peripheral blood B-cells after depletion with rituximab in patients with rheumatoid arthritis. *Arthritis Rheum* **54**: 613-20 (2006)
12. Tak PP *et al.* Baseline autoantibody status (RF, anti-CCP) and clinical response following the first treatment course with Rituximab. ACR 2006. Ref Type: Abstract
13. van Gestel AM *et al.* 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* **39**: 34-40 (1996)

14. van Oosterhout M *et al.* Clinical efficacy of infliximab plus methotrexate in DMARD naive and DMARD refractory rheumatoid arthritis is associated with decreased synovial expression of TNF alpha and IL18 but not CXCL12. *Ann Rheum Dis* **64**: 537-43 (2005)
15. Verpoort KN *et al.* Isotype distribution of anti-cyclic citrullinated peptide antibodies in undifferentiated arthritis and rheumatoid arthritis reflects an ongoing immune response. *Arthritis Rheum* **54**: 3799-808 (2006)
16. Brinkman DM *et al.* Vaccination with rabies to study the humoral and cellular immune response to a T-cell dependent neoantigen in man. *J Clin Immunol* **23**: 528-38 (2003)
17. van Esch WJ *et al.* Differential requirements for induction of total immunoglobulin and physiological rheumatoid factor production by human peripheral blood B-cells. *Clin Exp Immunol* **123**: 496-504 (2001)
18. Cohen SB *et al.* Rituximab for rheumatoid arthritis refractory 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* **54**: 2793-2806 (2006)
19. Emery P *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* **54**: 1390-1400 (2006)
20. Alwayn IP *et al.* Effects of specific anti-B and/or anti-plasma cell immunotherapy on antibody production in baboons: depletion of CD20- and CD22-positive B-cells does not result in significantly decreased production of anti-alphaGal antibody. *Xenotransplantation* **8**, 157-71 (2001)
21. Leandro MJ *et al*. Bone marrow B-lineage cells in patients with rheumatoid arthritis following rituximab therapy. *Rheumatology (Oxford)* (2006)
22. Vos K *et al.* Early effects of rituximab on the synovial cell infiltrate in patients with rheumatoid arthritis. *Arthritis Rheum* **56**: 772-8 (2007)
23. Cesano A *et al*. CD22 as a target of passive immunotherapy. *Semin Oncol* **30**:253-7 (2003)
24. Leung SO *et al.* Construction and characterization of a humanized, internalizing, B-cell (CD22)-specific, leukemia/lymphoma antibody, LL2. *Mol Immunol* **32**: 1413-27 (1995)
25. Dorken B *et al.* HD39 (B3), a B lineage-restricted antigen whose cell surface expression is limited to resting and activated human B lymphocytes. *J Immunol* **136**: 4470-9 (1986)
26. Campana D *et al.* Human B-cell development. I. Phenotypic differences of B lymphocytes in the bone marrow and peripheral lymphoid tissue. *J Immunol* **134**: 1524-30 (1985)
27. Bofill M *et al.* Human B-cell development. II. Subpopulations in the human fetus. *J Immunol* **134**: 1531-8 (1985)

28. Clark EA. CD22, a B-cell-specific receptor, mediates adhesion and signal transduction. *J Immunol* **150**: 4715-8 (1993)
29. Stamenkovic I *et al*. The B lymphocyte adhesion molecule CD22 interacts with leukocyte common antigen CD45RO on T-cells and alpha 2-6 sialyltransferase, CD75, on B-cells. *Cell* **66**: 1133-44 (1991)
30. Cambridge G *et al.* Serologic changes following B lymphocyte depletion therapy for rheumatoid arthritis. *Arthritis Rheum* **48**: 2146-54 (2003)
31. Cragg MS *et al*. The biology of CD20 and its potential as a target for mAb therapy. *Curr Dir Autoimmun* **8:** 140-74 (2005)
32. Withers DR *et al.* T-cell-dependent survival of CD20+ and CD20- plasma cells in human secondary lymphoid tissue. *Blood* (2007).
33. Masson-Bessiere C *et al.* In the rheumatoid pannus, anti-filaggrin autoantibodies are produced by local plasma cells and constitute a higher proportion of IgG than in synovial fluid and serum. *Clin Exp Immunol* **119**: 544-52 (2000)
34. Holers VM *et al.* Pathogenesis of autoantibodies. *Ann Rheum Dis* **65**: 10 (2006)
35. Williams DG *et al*. Clonal analysis of immunoglobulin mRNA in rheumatoid arthritis synovium: characterization of expanded IgG3 populations. *Eur J Immunol* **27**: 476-85 (1997)
36. Kim HJ *et al*. Plasma cell development in synovial germinal centers in patients with rheumatoid and reactive arthritis. *J Immunol* **162**: 3053-62 (1999)
37. Perry ME *et al.* Binucleated and multinucleated forms of plasma cells in synovia from patients with rheumatoid arthritis. *Rheumatol Int* **17**: 169-74 (1997)
38. Dechanet J *et al*. The ability of synoviocytes to support terminal differentiation of activated B-cells may explain plasma cell accumulation in rheumatoid synovium. *J Clin Invest* **95**: 456-63 (1995)
39. Jilani I *et al.* Transient down-modulation of CD20 by rituximab in patients with chronic lymphocytic leukemia. *Blood* **102**: 3514-20 (2003)
40. Pickartz T *et al.* Selection of B-cell chronic lymphocytic leukemia cell variants by therapy with anti-CD20 monoclonal antibody rituximab. *Exp Hematol* **29**: 1410-6 (2001)
41. Millar BC *et al*. Lymphotoxic activity of methyl prednisolone in vitro I. Comparative toxicity of methyl prednisolone in human cell lines of B and T origin. *Biochem Pharmacol* **36**: 831-7 (1987)