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Treatment with rituximab affects both the cellular and the humoral arm of the immune system in patients with SLE

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Abstract Herein we investigated how rituximab-induced B cell depletion affected leukocyte subpopulations and antibody titers in SLE patients. We focused our analysis on time points related to absence and return of B cells after depletion. A correlation was found between the baseline frequency and time to repopulation; the fewer B cells initially, the longer to their return. While the few B cells remaining after treatment were of memory, double-negative (IgD[−]CD27[−]), and CD5⁺ phenotype, the returning B cells were mainly naïve, indicating *de novo* production of B cells. Serum levels of IgG and antibodies against Ro52, Ro60, La44, measles and tetanus remained unchanged, while decreases in IgM, IgE, anti-dsDNA and anti-C1q antibodies were observed. Additionally, a significant increase in activated CD4⁺ and CD8⁺ T cells, as well as CD25^{bright}FOXP3⁺ regulatory T cells was observed. In conclusion, both the humoral and the cellular immune systems were affected by treatment with rituximab.

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Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune rheumatic disease, where one hallmark of the disease is the presence of autoantibodies against a series of nuclear antigens [1]. Disease manifestations include involvement of joints, skin, CNS and immune complex-mediated glomerulonephritis. Immune regulation is affected on several levels; defective apoptotic clearance and increased apoptosis [2],

diverted cytokine milieu [3–5], and defective cellular regulatory mechanisms [6,7]. The B cells are often seen as the central cause to disease pathogenesis [8,9]. Not only do they give rise to autoantibodies and immune complex formation involved in tissue damage, but they also have the capacity to act as antigen presenting cells activating and expanding autoreactive T cells which together with cytokine secretion create an overactivated immune system [9–11].

Since 1997, rituximab is an established treatment against non-Hodgkin lymphomas [12]. The target molecule, CD20, is solely expressed on B cells, which are depleted following infusion with rituximab. Since CD20 is not expressed on pro-B cells, rituximab does not deplete B cell progenitors, thereby allowing B cell reappearance after 4–12 months. Moreover,

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antibody-secreting plasma cells are also spared as they have downregulated their CD20 expression.

Lately, rituximab therapy has also been used successfully in the treatment of several autoimmune diseases [13], one of the first being rheumatoid arthritis (RA) [14]. Later on, an open study on SLE patients showed the treatment to be safe and effective with improvement of several disease manifestations [15]. These results have been confirmed also by others (reviewed by Sfrikakis, Boletis and Tsokos, and Thatayatikom and White [16,17]). Our group has recently demonstrated beneficial results of rituximab treatment in lupus patients with severe renal involvement [18,19].

In the present study, eleven SLE patients were followed for up to 30 months after rituximab therapy in order to analyze how different components of the immune system were affected by the treatment. The major aim was to investigate the relationship between the absence/presence of circulating B cells and other immune functions, such as presence of autoantibodies and presence and activation of T cells. We chose to study both the phenotypes of different subsets of B and T cells and antibody titers at B-cell-related time points, (1) before treatment, (2) at time of depletion, (3) at the time for repopulation of B cells in the circulation and (4) at the time when B cells had been present in the circulation for 2–6 months (Table 1). Serum levels of immunoglobulin (Ig)-G, -A, -M and -E, a panel of autoantibodies (IgG anti-dsDNA, -Ro52, -Ro60, -La44, -C1q), as well as protective IgG antibodies specific for tetanus toxoid and measles were measured. One major finding from this study was an increase of both activated and regulatory T cells. We could also see a heterogeneous effect on antibody titers, possibly indicating that different antibodies are produced by different B cell subsets.

Patients, material and methods

Patients and healthy controls

Eleven female patients with SLE (defined by the American College of Rheumatology criteria) [20] were recruited from the Rheumatology Clinic at the Karolinska University Hospital, Stockholm, Sweden. All patients had active disease and had failed conventional therapy including cyclophosphamide. Disease activity was determined by the Systemic

Lupus Activity Measure (SLAM) index of global lupus activity [21]. After therapy, the patients had a minimum of 6 months follow-up. Samples were collected at baseline and at 1, 2, 4, 6, 9, 12, 18, 24 and 30 months after treatment. Samples were also obtained from four non-treated healthy donors at three different time points to control for normal variations within the subpopulations analyzed. Five age-matched non-treated healthy subjects were used as controls for serology. This study was performed after human ethics approval from the Karolinska University Hospital. Informed consent was obtained from all contributing individuals. Table 2 provides a summary of the patients at baseline.

Treatment protocol

The therapy protocol was as previously described [18]. Briefly, the patients received four weekly infusions (375 mg/m²) of rituximab (mabthera, rituxan). Cyclophosphamide (0.5 g/m²) was included at the first and fourth occasion and corticosteroids were given throughout the treatment. After the fourth infusion, no other therapy than corticosteroids was given until repopulation occurred.

Cell separation and flow cytometry

Peripheral blood samples were collected in Na-Heparin Vacutainer tubes. Mononuclear cells were prepared by Ficoll separation (Ficoll-Paque, Pharmacia, Uppsala, Sweden). For phenotypical characterization cells were stained with antibodies from Pharmingen and Becton Dickinson (New Jersey, USA) against the following cell surface markers: CD3-FITC (UCHT1), CD3-PerCP (SK7), CD4-PerCP/-APC (SK3), CD5-PE (L17F12), CD8-FITC (SK1), CD19-PerCP/-APC (SJ25C1), CD20-PerCP (L27), CD25-PE (2A3), CD27-PE (M-T271), CD138-APC (MI15), IgD-FITC (IA6-2). Irrelevant mouse IgG1 and 2a were used as isotype controls. Detection of intracellular FOXP3/scurfin was performed with the anti-human FOXP3-APC (PCH101) staining set from eBiosciences (cat. no. 77-5776, California, USA) according to instructions from the manufacturer. The stained cells were analyzed by collecting 100,000 events within the live gate as defined by forward and side scatter with flow cytometry on a FACS Calibur (Becton Dickinson, New Jersey, USA) and analyzed with Summit software from Dako Cytomation (Glostrup, Denmark).

The clinical immunology laboratory at Karolinska University Hospital analyzed total numbers and frequencies of the different leukocyte populations according to clinical routine.

The range for normal values was either provided by the clinical immunology laboratory or from analysis of healthy subjects analyzed in our laboratory.

Serum concentration of antibodies

Total immunoglobulins

Levels of IgG, IgA and IgM were measured and determined by nephelometry and immunodiffusion at the clinical immunology laboratory at Karolinska University Hospital.

Serum IgE was measured by an enzyme-linked immunosorbent assay (ELISA) kit from Alpha Diagnostic International, ADI (cat. no. 1800, Texas, USA) according to the instructions from the manufacturer.

Table 1 B-cell-related time points

B-cell-related time point	B cell status	Comment
Baseline	Before rituximab treatment	Sample taken shortly before first rituximab infusion
Depletion	B cells <0.5% of lymphocytes and <0.01 × 10 ⁹ /l blood	Sample taken 2 months after fourth infusion
Repopulation	B cells >1.0% of lymphocytes	Occurred between 4 and 12 months after fourth infusion
Recovery	The next following sample after repopulation	Taken 2–6 months after repopulation

Table 2 Baseline description of patients

Patient no.	Age (years)	Disease duration (years)	Initial dose of prednisone (mg) ^a	Disease manifestations (previous and ongoing)	Prior immunosuppressive therapies	Autoantibodies at baseline ^b
1	27	11	40.0	Nephritis, cutaneous, serositis, vasculitis	CyX, CyA, MMF	dsDNA, C1q
2	56	20	15.0	Nephritis, cutaneous, serositis, arthritis	CyX, AM, MTX, AZA, Ivlg, CyA	dsDNA, La44,
3	32	7	15.0	Nephritis, myositis, antiphospholipid antibody syndrome, Quincke edema	CyX, AZA, CyA	Ro52, Ro60
4	26	10	15.0	Nephritis, cutaneous, serositis, arthritis	CyX, AZA	dsDNA, C1q
5	50	4	12.5	Arthritis, Sjögrens syndrome, Raynaud, serositis, hematological	CyX, AZA, CyA, Leflunomide	dsDNA
6	33	8	10.0	Nephritis, cutaneous, hematological	CyX, CyA	dsDNA, C1q
7	32	8	12.5	Nephritis, cutaneous, arthritis, CNS	CyX, AM, MMF, AZA, MTX	dsDNA, Ro52, Ro60, C1q
8	19	3	15.0	Nephritis, cutaneous, arthritis, hematological	CyX, AM, MMF, AZA, MTX	dsDNA, C1q
9	35	9	20.0	Nephritis, photo sensitivity, arthritis, CNS, vasculitis	AM, MMF, AZA, CyA, MTX, Ivlg	dsDNA, La44, C1q
10	55	2	10.0	Arthritis, subacute cutaneous LE, serositis, CNS	CyX, AM	none
11	50	16	15.0	Arthritis, puffy hands, hematological	MTX, AM, CyA, AZA,	Ro52, Ro60

CyX indicates cyclophosphamide; CyA, cyclosporine A; MMF, mycophenolate mofetil; AM, antimalarials; MTX, methotrexate; AZA, azathioprine; Ivlg, intravenous immunoglobulins.

^a T. Jonsdottir et al. submitted manuscript with a thorough clinical study of the same patient cohort.

^b Autoantibodies listed here are only those which are investigated in this study. dsDNA indicates anti-double-stranded DNA; C1q, anti-C1q; La44, anti-La44; Ro52, anti-Ro52; Ro60, anti-Ro60.

Antibodies against measles and tetanus toxoid

To measure IgG antibodies against measles we used Enzygnost Anti-Measles ELISA kit (cat. no. OWLN155, Dade Behring Marburg GmbH, Marburg, Germany). For measurements of IgG against tetanus toxoid an ELISA kit from Institut Virion/Serion GmbH (cat. no. ESR108G, Würzburg, Germany) was used. Both ELISAs were performed according to instructions from the manufacturer.

Autoantibodies

IgG anti-dsDNA was measured at the clinical immunology laboratory at Karolinska University Hospital by fluorescence enzyme immuno-assay.

IgG anti-C1q was measured by ELISA at the clinical immunology laboratory at Lund University Hospital, Sweden.

An inhouse ELISA was used to measure IgG anti-Ro52, -Ro60, and -La44 as previously described [22]. Briefly, serum was added to Ro52, Ro60 and La44 coated wells on a medium binding plate (Corning Inc., New York, USA). Bound antibodies were detected with anti-IgG antibodies conjugated with alkaline phosphatase (cat. no. 3310-1, Mabtech, Nacka, Sweden). The absorbance was measured at 405 nm after addition of phosphatase substrate tablets (Sigma, Missouri, USA). Serum from a patient with high titers of IgG antibodies to Ro52, Ro60, and La44 was used as a positive control.

Statistical analysis

Statistical analysis was performed using Graphpad Prism 3.03 (Graphpad Software Inc., California, USA) and Statistica 7.1 (StatSoft Inc., Oklahoma, USA). For comparison of paired samples, Wilcoxon-matched pairs test for nonparametric data was used. Pearson correlation of parametric data was used for analysis of correlation.

Results and discussion

In order to elucidate mechanisms of B cell depletion in patients with SLE a longitudinal analysis of cellular and serum-based parameters was performed on samples from eleven patients with refractory SLE (Table 2). All patients improved clinically upon rituximab treatment, which is reflected in the decrease in SLAM score over time (Fig. 1A) and also described in a separate manuscript in preparation (T. Jonsdottir et al.). In all patients circulating B cells were depleted upon rituximab treatment (Fig. 1C, Table 3). Fig. 1C also depicts for how many months each patient has been followed immunologically. The large inter-individual variation in time between peripheral B cell depletion and B cell repopulation prompted us to define B-cell-related time points for immunological comparisons between the patients.

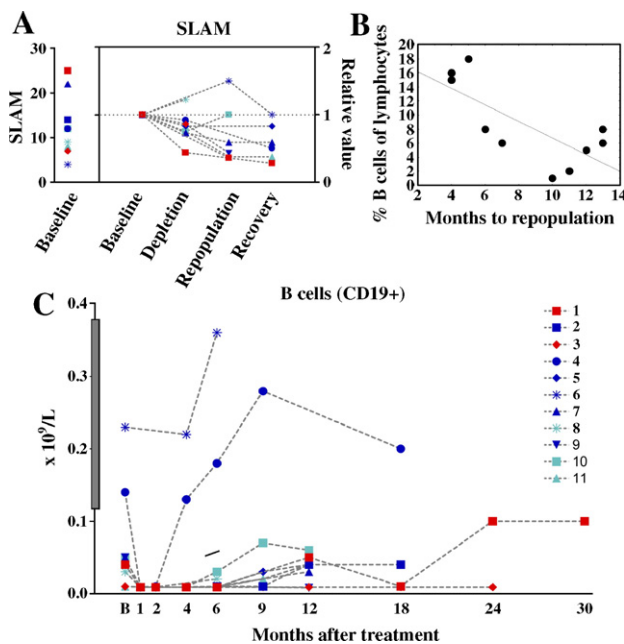


Figure 1 B cells and disease index before and after rituximab therapy. (A) SLAM scores, a measure of disease activity, at B-cell-related time points. To the left are the SLAM scores for each patient at baseline (before treatment) illustrated. The diagram to the right illustrates relative changes after treatment; baseline values are set to 1 and following samples are compared to baseline value (relative value = sample X/baseline sample). In the two patients where a relative increase in SLAM score was observed, the values varied between 4 and 6, and 9 and 11, respectively. (B) Negative correlation between the frequency of B cells and time point of B cell repopulation. On the x-axis is the number of months from rituximab treatment to B cell return in the circulation, and on the y-axis the frequency of circulating B cells within the lymphocyte population is indicated. Pearson $r = -0.74$, $p = 0.015$. (C) All patients were depleted of peripheral B cells after rituximab therapy. The x-axis depicts baseline, B, and time after treatment (1–30 months). The normal range of B cells in the circulation is indicated by the grey bar on the y-axis. Median time for repopulation was 7.5 months. Each patient is represented by one colored symbol in panels A and C.

As described in Table 1 we chose to use the following time points for our analysis: (1) before onset of treatment, (2) at time of depletion, (3) at time of repopulation, and (4) at time of recovery of cells in the circulation. Repopulation is when there is a significant frequency of B cells within the lymphocyte population, $>1\%$, which can contribute immunologically to immune reactions. As can be seen in Table 2, all but two patients had previously been treated with cyclophosphamide, thus the baseline levels include immunological effects of that drug.

Effect of rituximab on numbers and frequency of circulating B cells

As described above a successful depletion of circulating B cells was observed in all patients after four weekly doses of rituximab infusion (Fig. 1C, Table 3). The median time to repopulation was 7.5 months (range 4–12 months), while one

patient had not regained her B cells after 24 months. In the majority of patients (9/11) the total number of circulating B cells was lower than in healthy subjects even before initiation of rituximab treatment, possibly explaining the complete deletion of circulating B cells in all patients, in contrast to the rituximab-treated SLE patients described by Looney et al. [23]. Interestingly, a correlation was found between frequency of B cells before treatment and time point of return in the circulation (Fig. 1B, Pearson $r = -0.74$, $p = 0.015$), i.e., individuals with a more pronounced B cell lymphopenia had a slower return of B cells to the circulation. This knowledge may help us to predict the time of repopulation of B cells in single individuals. It may also be a future tool to optimize dosage of rituximab in single patients, where patients with a high frequency of B cells before treatment might need higher doses or a second treatment with rituximab in order to achieve a better depletion of B cells in other compartments than the circulation.

B cell subpopulations

Before investigating the reappearing B cells (repopulation), we characterized the residual B cells directly after therapy (depletion). Even if the patients were considered as B cell depleted at this time point ($<0.5\%$ of lymphocytes and $<0.01 \times 10^9/\text{l}$ blood) acquisition of a large number of mononuclear cells in the flow cytometer allowed detection of the remaining B cells and a distinction of different subsets, as has also been described by Anolik et al. in their SLE cohort and by Leandro et al. in RA [24,25]. A summary of the distribution of phenotypes among the few cells remaining after depletion is seen in Table 4, they were CD27+CD19+ memory B cells, CD19+CD27^{high} plasmablasts, CD27–IgD–CD19+ B cells and CD5+CD19+ B cells, see also Fig. 2, columns “depletion”. Upon repopulation, the frequencies of both CD27+CD19+ memory B cells and of CD27–IgD–CD19+ B cells were reduced below their original frequencies, while CD27^{high} plasmablasts and CD5+CD19+ B cells were expanded at repopulation. Our data indicate that the B cells remaining after treatment are subpopulations more resistant to CD20 depletion, and/or alternatively cells extravasating from the lymphoid tissues [26]. Interestingly, these non-depleted populations were in a minority upon repopulation suggesting that they only perform limited homeostatic proliferation in the absence of the other subsets, and repopulation is thus predominantly dependent on the regeneration from B cell precursors. Rouzière et al. [27] have investigated changes in the IgH-repertoire in two rituximab-treated RA patients. They conclude that a transient B cell depletion by rituximab regenerates a diverse and polyclonal heavy-chain repertoire characterized by highly mutated B cells. Their results might reflect the expansion of various B cell subpopulations during depletion and repopulation.

In order to study the reappearing B cells after therapy, we analyzed the frequencies of naive and memory B cells. Despite the reduced number of B cells already before treatment (Fig. 1C) in all patients but one, the frequency of memory B cells (CD19+CD27+) was within the normal range (Fig. 2A). At repopulation, the frequency of memory B cells decreased as expected, and was less than 30% of the CD19+ B lymphocytes, which equals approximately half of the

Table 3 Number of lymphocytes, T cells and B cells per patient at B-cell-related time points

	Patient	1	2	3	4	5	6	7	8	9	10	11
Lymphocytes cells $\times 10^9/l$	Basel.	0.82	0.73	0.48	0.90	0.32	1.51	0.28	0.53	0.64	0.65	0.50
	Depl.	0.85	0.70	1.79	0.70	0.22	n.d.	0.66	0.41	0.66	0.29	0.39
	Repop.	1.09	1.04		1.03	0.35	1.96	0.17	n.d.	1.01	0.65	0.67
	Recov.	0.53	0.55		1.13	0.39	2.16	0.31	n.d.		0.64	0.66
T cells CD3+ cells $\times 10^9/l$	Basel.	0.69	0.48	0.41	0.69	0.21	1.14	0.17	0.35	0.52	0.56	0.31
	Depl.	0.77	0.60	1.67	0.62	0.17	n.d.	0.60	0.35	0.58	0.24	0.29
	Repop.	0.93	0.86		0.83	0.27	1.51	0.11	n.d.	0.83	0.45	0.52
	Recov.	0.45	0.43		0.86	0.30	1.52	0.21	n.d.		0.48	0.46
B cells CD19+ cells $\times 10^9/l$	Basel.	0.04	0.04	0.01	0.14	0.01	0.23	0.05	0.03	0.05	0.05	0.01
	Depl.	bdl	bdl	bdl	0.01	bdl	n.d.	bdl	bdl	bdl	bdl	bdl
	Repop.	0.05	0.04		0.13	0.03	0.22	0.01	n.d.	0.08	0.03	0.02
	Recov.	0.01	0.04		0.18	0.04	0.36	0.03	n.d.		0.07	0.04
%CD19+ B cells of lymphocytes	Basel.	5	6	2	16	2	15	18	6	8	8	1
	Depl.	bdl	bdl	bdl	1	bdl	n.d.	bdl	bdl	bdl	bdl	bdl
	Repop.	5	4		13	8	11	3	4	8	4	2
	Recov.	2	6		16	9	18	10	n.d.		12	5

Basel. = Baseline; Depl. = Depletion; Repop. = Repopulation; Recov., Recovery; bdl, below detection limit; n.d. = not determined.

baseline values. The memory B cells remained lower both in frequency (median 77% of baseline) and number (median 73% of baseline, data not shown) in the majority of the patients during recovery, confirming a continued output of naïve B cells. This is also proposed by other studies: Leandro et al. have characterized repopulating B cells in patients with RA as immature (CD19+IgD+CD38^{high}CD10^{low}CD24^{high}) and Anolik et al. suggest reconstitution of peripheral blood B cells in patients with SLE to be of immature or transitional phenotype [25,28].

Anolik et al. recently described that a double-negative B cell population (CD19+CD27–IgD–) was expanded in SLE patients and adjusted back to normal after rituximab treatment [24]. In our cohort all patients had a higher frequency of CD19+CD27–IgD– double-negative cells before treatment (median 11.7%, range 4.2%–44.7%, Fig. 2B) as compared to the healthy controls (median 4% of B cells). At recovery, the frequency was reduced to 34% of baseline (range 0.9–63% of B cells). Our data thus confirm the results of Anolik et al., with enrichment during depletion but no disproportional expansion at repopulation.

In mice, B cells expressing CD5 on their surface are considered as a cell lineage of their own, B-1 B cells. These cells have been shown to secrete low affinity IgM natural antibodies, IL-10, and have been associated with autoimmune disease (reviewed in Herzenberg, and Martin and Kearney [29,30]). However, evidence suggests that B1 B cells are not the source of autoantibodies in some mouse models

of autoimmune syndrome [31–33]. In humans, it is debated whether CD5+ B cells are B-1 B cells or not. It has been shown that both CD5+ and CD5– B cells produce antibodies against autoantigens as well as low affinity IgM antibodies and IL-10 [34,35]. Also, CD5 can be transiently upregulated on B cells following activation [36,37]. Böhm et al. recently demonstrated that SLE patients have an increased frequency of these cells [38] and since the CD5+ B cell population was partly resistant to rituximab treatment in our cohort (Table 4) we were interested to study them at all B-cell-related time points.

At baseline the frequency of CD5+ B cells varied, but interestingly at repopulation they increased 3.2-fold among the CD19+ B cells (Fig. 2C) and finally tended to stabilize at recovery to 1.3-fold higher than baseline. Also the total number of CD5+ B cells doubled (2.2-fold) at repopulation and was 1.8-fold at recovery (data not shown). Possible explanations for these results could be a resistance to rituximab-induced B cell depletion or a faster recovery of CD5+ B cells as compared to CD5– B cells. Alternatively, the treatment itself could indirectly or directly induce this phenotype. In studies on rituximab-treated dialysis patients awaiting renal transplantation and rituximab-treated RA patients CD5+CD19+ B cells were the first B cells returning to the periphery, supporting the concept that they recover faster than CD5– B cells [25,39]. Further phenotypic analysis is needed in order to investigate the cell lineage of these cells. Such an analysis could also exclude the possibility that the CD5+ B cells are plasmablasts with low/no expression of CD20, hence not affected by rituximab.

Plasmablasts (CD27^{high}CD19+) are found in the circulation of healthy subjects (Fig. 2D). However, in SLE patients with active disease this population is known to be increased [40]. In our cohort, half of the patients had high levels (6.9%–37.5%) of these cells while the other patients were within the normal range (Fig. 2D). At all time points after treatment the frequency of plasmablasts was increased, 3.6-fold at depletion, 2.8-fold at repopulation, and still 2.3-fold at recovery (all median values). In contrast to data by Anolik et al.,

Table 4 Residual B cells during depletion

B cell subpopulation ^a	Median (%)	Min-Max (%)
CD27+ of CD19+	50	22.4–95.2
CD27 ^{high} of CD19+	3.1	0–76.2
IgD–CD27– of CD19+	47.9	7.8–76.3
CD5+ of CD19+	56.6	7.9–86.7

^a B cells were either stained for CD5 or CD27, thus these populations can be overlapping.

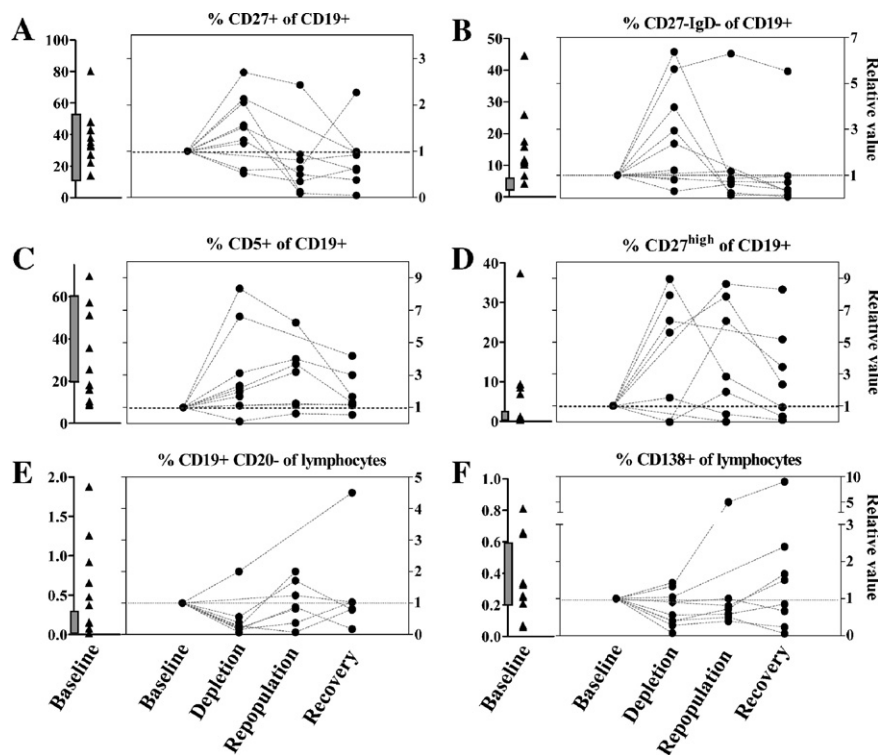


Figure 2 Frequency of B cell subpopulations at B-cell-related time points. To the left of each figure the normal range in frequency is indicated as grey bars, each triangle represents one patient at baseline. To the right are frequencies of the indicated populations at the B-cell-related time points presented, where the baseline value is set to 1 for each patient and following samples are compared to baseline value (relative value=sample X/baseline sample). For each figure the analyzed cell population is indicated above the figure, the relative values to the right of the figure. (A) Frequency of memory B cells within CD19+ B cells, (B) double-negative (IgD-CD27-) B cells within CD19+ B cells, (C) CD5+ cells within CD19+ B cells, (D) CD27^{high} cells within CD19+ B cells, (E) CD19+ CD20- B cells within lymphocytes, (F) CD138+ plasma cells within lymphocytes.

who described a decrease in CD38+CD19^{low} plasma cells/plasmablasts [24], only in a minority of our patients did plasmablasts decrease in the circulation (Table 5).

CD20-negative plasma cells are not directly affected by rituximab treatment. But the possibility that these cells fill immunological niches when CD20+ B cells are gone, prompted us to analyze if there was an increased output of such cells from the bone marrow upon B cell depletion. We therefore measured the frequency of circulating CD138+ plasma cells. Before treatment half of the patients had similar levels of plasma cells as healthy controls, three patients had higher levels and two patients had lower levels (Fig. 2F). A trend towards a lowered frequency during depletion and repopulation was observed (median 56% and 73% of baseline respectively, Fig. 2F). This decrease in plasma cells could be due to a delay in the development of such cells, alternatively a preferred homing to peripheral tissues and bone marrow [41].

Effects of rituximab on antibody levels in serum

In view of the significant deletion of circulating B cells and previously published results on changing antibody levels after rituximab treatment in SLE patients [15,23,24,42] we performed a detailed analysis of circulating antibodies. To increase our understanding of which antibody producing cells are the main source of different antibodies, we chose a wide

repertoire of antigen specificities. In addition to total levels of immunoglobulin isotypes we focused on three different types of antigen-specific antibodies; (i) potential pathogenic and disease activity-associated autoantibodies, (ii) potential pathogenic autoantibodies not associated with disease activity, and (iii) protective antibodies.

Changes in serum levels of total immunoglobulins

Serum levels of immunoglobulins G, A, M, and E were measured before and repeatedly after therapy. Presented in Figs. 3A-D are both the baseline titers and the relative changes at the B-cell-related time points.

No significant changes were observed in levels of total IgG, both increases and decreases were observed in individual

Table 5 Changed frequency of plasmablasts compared to baseline

B-cell-related time point	No. of patients with lower frequency	No. of patients with same frequency	No. of patients with higher frequency
Depletion	3	1	4
Repopulation	2	0	5
Recovery	2	1	4

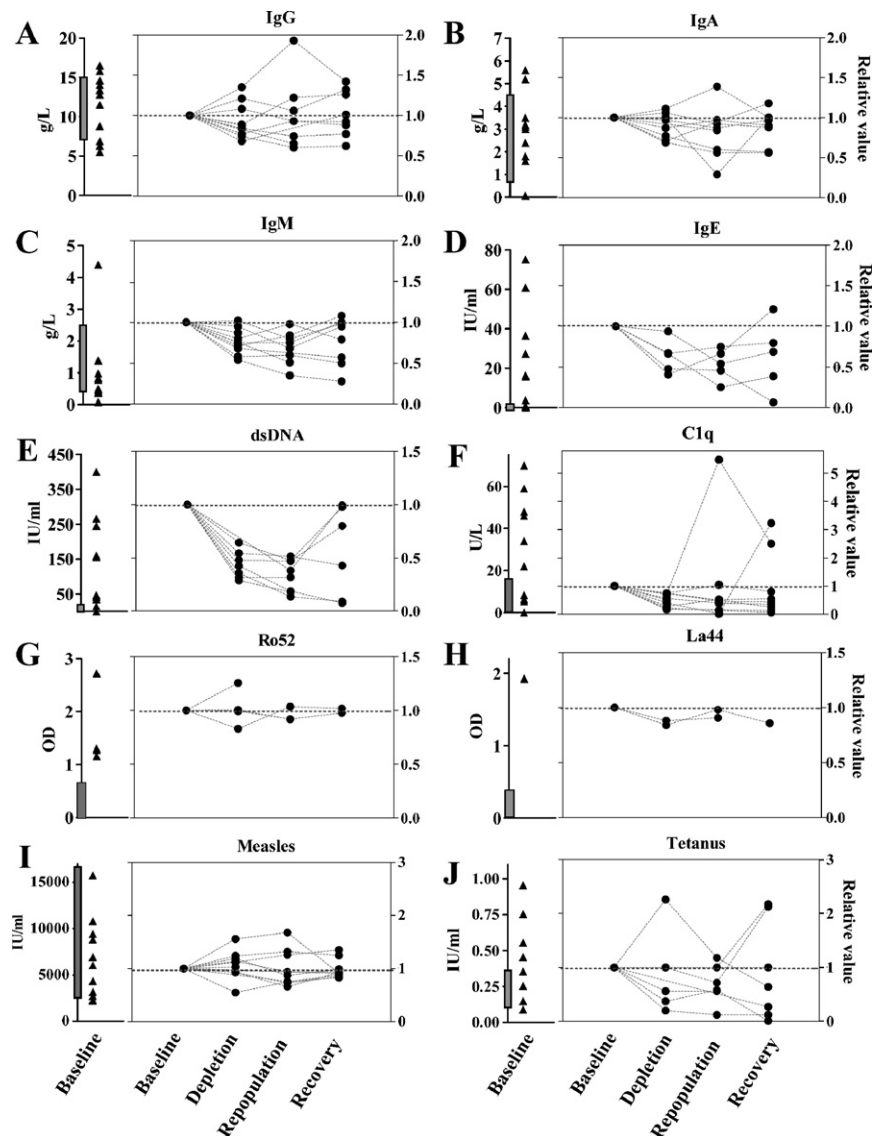


Figure 3 Serum antibodies at B-cell-related time points. Both total and specific antibody titers were measured at the B-cell-related time points. To the left of each figure the normal range for healthy subjects is indicated as grey bars, each triangle represent one patient at baseline. To the right are levels of the indicated antibody type at the B-cell-related time points presented, where the baseline value is set to 1 for each patient and relative changes for each patient can be followed as indicated on the y-axis to the right (relative change = sample X/baseline sample). (A-D) Total Ig levels: (A) IgG, (B) IgA, (C) IgM, and (D) IgE. (E-J) Specific antibody titers: (E) disease activity-associated IgG anti-dsDNA autoantibodies ($n=8$), (F) disease activity-associated IgG anti-C1q autoantibodies ($n=6$), (G) IgG anti-Ro52 autoantibodies ($n=3$), (H) IgG anti-La44 autoantibodies ($n=2$), (I) IgG anti-measles, and (J) IgG anti-tetanus toxoid antibody titers.

patients. The titers remained within normal range. This confirms earlier studies both in SLE and RA [14,23,43].

In contrast to IgG, IgM decreased rapidly during B cell depletion to a median 75% of baseline values. The levels remained low at repopulation (median 72% of baseline) and started to slowly increase at recovery (median 87% of baseline). This has not been observed previously, neither in SLE nor RA patients [15,44], but in patients with B cell lymphoma [45]. For IgA levels, all patients but one showed decreased levels at repopulation (median 84% of baseline), however levels remained within normal range as also demonstrated by Leandro et al. [15].

The IgE titers also decreased after rituximab treatment. Like IgM, the decrease was seen already at depletion with a median 66% of baseline values in the five patients who had IgE titers at baseline. The levels continued to decrease until repopulation to a median 54% of baseline, and at recovery they had increased to a median 69% of baseline values. Increased IgE levels and antinuclear IgE autoantibodies appear to occur without specific clinical signs of allergy or production of anti-allergen IgE antibodies in SLE [46,47].

From these data combined with discussions on long-lived plasma cells [48,49] we speculate that most of the IgG and IgA are produced by long-lived plasma cells, while IgM and IgE

antibodies primarily are produced by plasmablasts and short-lived plasma cells.

Selective changes in antibody titers to different antigens

In parallel to the analysis of total levels of circulating immunoglobulins, we analyzed a panel of autoantigen-specific IgG antibodies. We included anti-double-stranded DNA (dsDNA) antibodies and anti-C1q antibodies, which both have been reported to follow disease activity in SLE patients, and also anti-Ro52, -Ro60 and -La44 antibodies, which if present, are considered constant despite changes in disease activity [50-53]. Each patient's unique antibody profile at baseline is presented in Table 2.

Anti-dsDNA antibodies were detected in 8/11 patients before onset of therapy, and these decreased rapidly already at depletion from 158 to 45 IU/ml (median 43% of baseline, $p=0.016$). The decrease was most prominent at time of B cell repopulation with only 35% of the specific antibodies in the circulation ($p=0.031$) (Fig. 3E). To further emphasize that the total IgG levels do not change while the anti-dsDNA titers do, we also show the longitudinal data in Fig. 4. These data are in concordance with the longitudinal follow up of SLE patients by Leandro et al. [42].

Six of eight patients with nephritis had measurable levels of anti-C1q antibodies at baseline (range 22-70 U/l). Like anti-dsDNA antibodies, the titers decreased rapidly to 25% of baseline at depletion (median 12 U/l, range 6-33 U/l). They remained low during repopulation (43% of baseline) and recovery (31% of baseline) (Fig. 3F). Generally, each patient has her unique autoantibody profile, and levels but not the presence of specific antibodies changed upon treatment. One exception being a patient without signs of nephritis nor anti-C1q antibodies before rituximab infusion, but surprisingly, after treatment anti-C1q antibodies were detected (Fig. 3F). This patient was lymphopenic and remained so throughout her follow-up. This parallels a finding by Armstrong et al., who demonstrates a correlation between anti-C1q antibodies and lymphopenia in hematological patients without nephritis [54]. These similar changes raised the question if other autoantibody titers also change upon

treatment. We therefore measured titers of Ro and La antibodies. Interestingly, in contrast to the titers of anti-dsDNA and anti-C1q, no significant changes of anti-Ro52 (Fig. 3G), anti-La44 (Fig. 3H), and anti-Ro60 (data not shown) were detected in the patients, who had such antibodies initially. Our study, which is the first to investigate possible effect from rituximab treatment on antibodies against C1q, Ro52, Ro60 and La44, confirms studies demonstrating that titers of anti-C1q correlate with disease activity. The stable levels of anti-Ro and -La antibodies observed in our small number of patients with such antibodies present needs to be extended in order to confirm that such antibody titers are independent of disease activity in rituximab-treated SLE patients. There are studies in patients with Sjogren's syndrome also indicating that anti-SSA and -SSB antibodies remain after rituximab treatment [55,56]. Our results add information to the ongoing discussion on short- and long-lived plasma cells and their different antigen specificities [48,57-59].

We decided also to investigate if other antibodies were affected by B cell depletion, and thus measured antibodies important for protective immunity. The antibody titers against measles, which provide us with life long immunity, did not change over the time period investigated (Fig. 3I). In contrast, an apparent decline in titers of IgG anti-tetanus was seen in many patients, but larger cohorts are needed to test if this is a general finding after rituximab treatment in SLE patients (Fig. 3J). These data only partly parallel those in RA, where rituximab induced a decrease in rheumatoid factor, while anti-tetanus antibody titers remained unchanged [44]. However, we believe that extended longitudinal follow-ups of antibody titers important for long-term immunity against pathogens are needed after rituximab treatment. The differential effects of rituximab treatment on antibody production to different antigens warrant additional studies.

An important consideration is that B cell depletion could be variable for different lymphoid organs, such as lymph nodes, spleen, and bone marrow. Limited information on the *in vivo* effects of anti-CD20 antibodies in primary lymphoid organs exists in animals, but so far not in any models of SLE. B

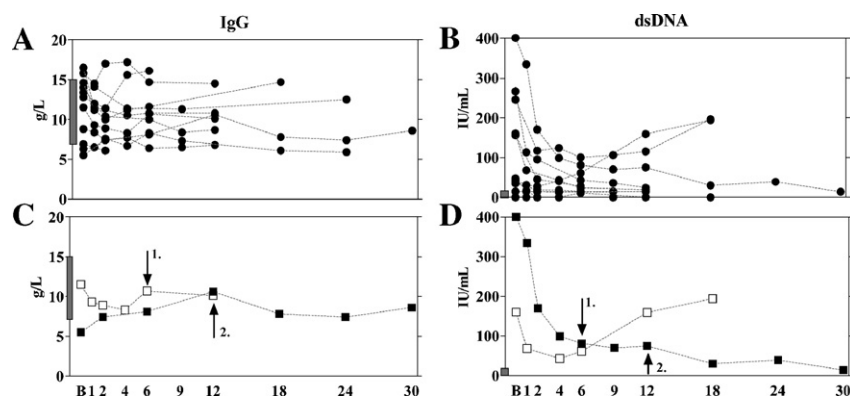


Figure 4 Longitudinal serum levels of total IgG and dsDNA before and after rituximab treatment. (A) Serum levels of total IgG and (B) anti-dsDNA were followed in all eleven patients longitudinally after rituximab therapy. Normal IgG levels are indicated by the grey bar on the y-axis, time points of sampling in months on the x-axis. The lower diagrams exemplifies two patients with regard to (C) total IgG and (D) anti-dsDNA in relation to the time point of B cell repopulation (indicated by arrows: patient 1, open symbols and patient 2, filled symbols).

cell depletion in cynomolgus monkeys demonstrated a variable diminishment (69-87% depletion of B cells) in inguinal lymphnodes and 74-95% depletion in bone marrow after four weekly injections [60]. Also, studies performed on mice transgenic for human CD20 showed that approximately 50% of the marginal zone B cells and most of the germinal center B cells residing in Peyer's patches remained after rituximab treatment [61].

Thus, depletion of B cells in the circulation does not necessarily indicate successful depletion in tissue. This therapy could be used as a model to increase our understanding of different antibody-producing cells by analyzing an even broader panel of antibodies longitudinally in addition to antibody production on a cellular level.

Leukocytes and subpopulations thereof

As described above, the total numbers of circulating B cells decreased temporarily in response to rituximab treatment (Fig. 1C). In order to identify changes that might affect also other cells of the immune system we analyzed different subpopulations of leukocytes. With regard to total number of leukocytes, neutrophils and NK cells no specific changes were observed; individual increases and decreases were measured after treatment (Figs. 5A, B and D, Table 3). The total number of circulating monocytes remained within the normal range at all time points, although increases were measured early after treatment in all but one patient, possibly indicating a compensation for the loss of antigen presenting B cells (Fig. 5C). A trend towards an increase in

number of lymphocytes after rituximab treatment was observed, however, most patients remained below the normal range (Fig. 5E). As expected from the lymphopenic state of the patients, ten of eleven patients had lower than normal number of T cells at start of rituximab treatment (Fig. 5F). Only two patients showed a substantial T cell increase. Overall, the T cells demonstrated only a slight temporary increase at repopulation (median 1.3-fold of baseline), and these data are in concordance with a previous publication on RA patients [25]. Sfrikakis et al. studied T cells in rituximab-treated SLE patients and demonstrated an increase in T cells, however it was observed at time of clinical response making a direct comparison to our data difficult [62].

T cell subpopulations

Although no significant changes in total numbers of T cells were seen, additional investigations revealed important changes of T cell subsets following B cell depletion in our patient cohort (Table 3). Both low frequencies (Fig. 6A) and low numbers (data not shown) of CD4+ T cells were observed, but no obvious changes after treatment could be measured.

There are a number of reports describing that a disproportional high frequency of CD4+ T cells are activated in SLE patients (reviewed by Kyttaris and Tsokos) [63]. Traditionally, CD25 has often been used to identify activated T cells, and high expression of CD25 is a marker for regulatory T cells [64]. In the healthy subjects 16-37% of the CD4+ T cells expressed CD25. Five of ten patients had a higher frequency of these cells at baseline (>37%, Fig. 6B). Surprisingly, at repopulation the frequency of these cells

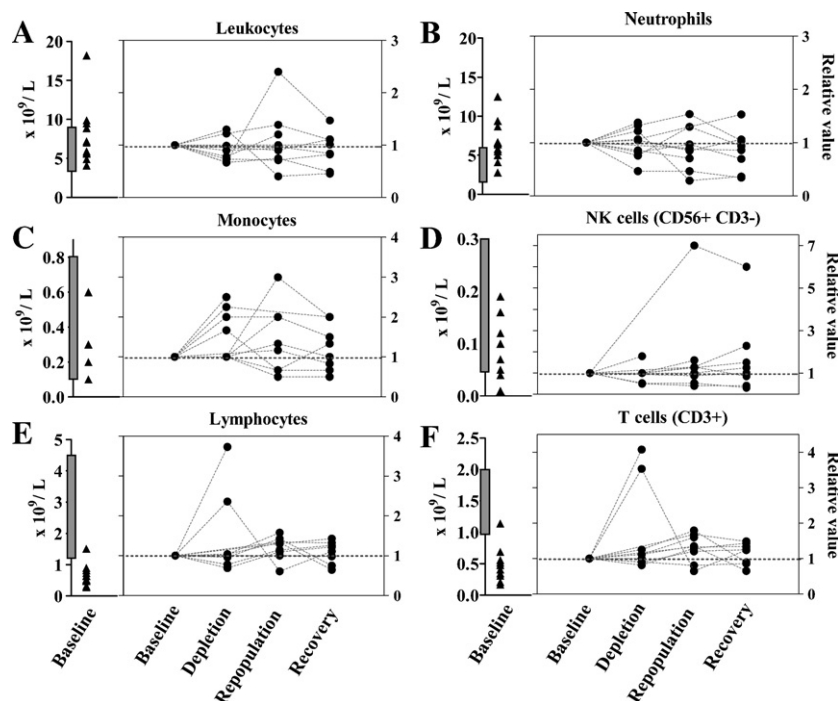


Figure 5 Populations of leukocytes at B-cell-related time points. To the left of each figure the normal range in cell number is indicated as grey bars, each triangle represents one patient at baseline. To the right are total number of the indicated populations at the B-cell-related time points presented, where the baseline value is set to 1 for each patient and relative changes indicated on the y-axis to the right of each figure (relative change=sample X/baseline sample). No changes in total number of (A) leukocytes, (B) neutrophils, (D) NK cells, (E) lymphocytes or (F) T cells were observed after rituximab treatment. A trend towards an increase in (C) monocytes was observed in the majority of patients at depletion.

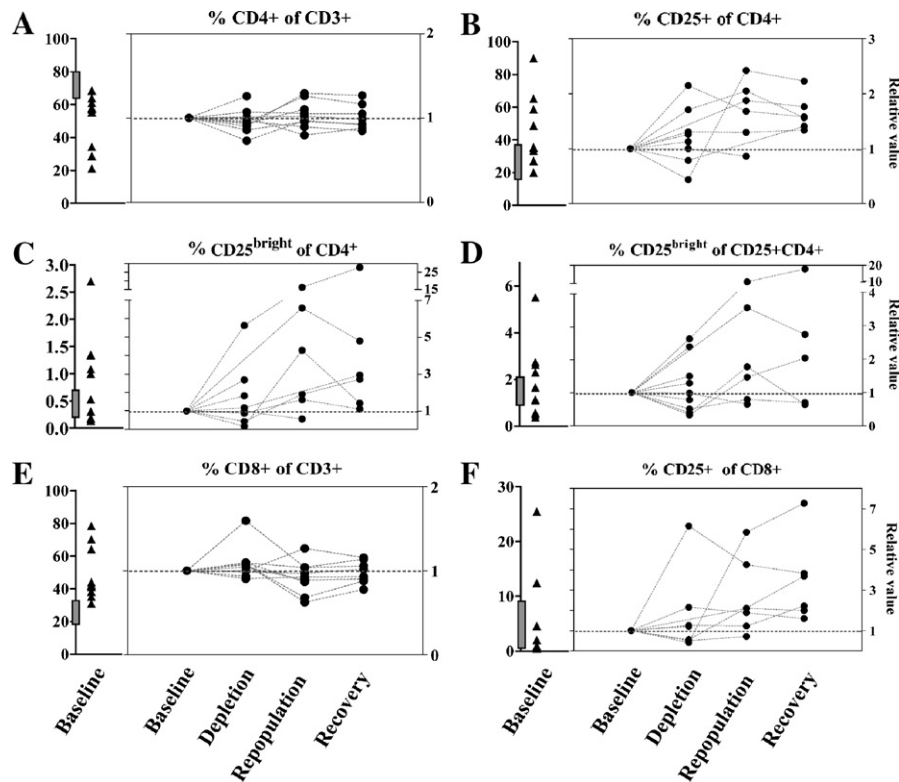


Figure 6 Frequency of T cell populations at B-cell-related time points. To the left of each figure the normal range in frequency is indicated as grey bars, each triangle represents one patient at baseline. To the right are frequencies of the indicated populations at the B-cell-related time points presented, where the baseline value is set to 1 for each patient and relative values indicated on the y-axis to the right of each figure (relative change=sample X/baseline sample). For each figure (A-F) the analyzed cell population is indicated above the figure.

had increased in all but one patient, and at recovery all samples analyzed demonstrated an increase (median 1.6-fold). Importantly, at recovery not only the frequency but also the number of activated CD25+CD4+ T cells had increased 1.6-fold (data not shown). The proportion of activated T cells has been shown to decrease in another study [62], however, they used other markers of activation and had a different clinical setting.

When comparing regulatory T cells at the B-cell-related time points, both the frequency of CD25^{bright} regulatory T cells (Fig. 6C) and the number (data not shown) had increased in all patients at recovery (median 2.8-fold). Regulatory T cells have been demonstrated to be important players in the control of experimental autoimmunity [64]. In order to relate the frequency of regulatory T cells to the frequency of activated CD4+ T cells, we calculated the ratio of CD25^{bright}CD4+ T cells to the total of cells within the CD25+ gate, which comprises both activated and regulatory T cells. Already at baseline, four of ten patients had higher than normal amount of CD25^{bright} cells within the CD25+ T cell population. After treatment, a tendency towards an increase at repopulation and recovery was observed (median 1.6 and 2.0 respectively, Fig. 6D). These data give one possible explanation as to why patients improved despite an increased frequency of activated CD4+ T cells: the suppressive effect of regulatory T cells can overrule the activation, and thus immune homeostasis is re-established upon treatment. In order to support our data that CD25^{bright} expression

mainly identifies regulatory T cells also in a highly activated immune system, flow cytometric staining of FOXP3, a transcription factor specific for functional regulatory T cells [65], was performed on samples obtained before treatment and at repopulation from two patients. As shown

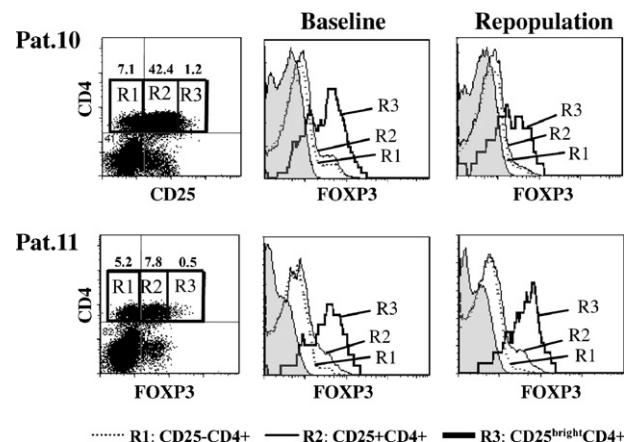


Figure 7 CD25^{bright}CD4+ T cells express FOXP3. Each row represents one patient. Left column: FACS dot plots with gates for CD25- (R1), CD25^{int} (R2) and CD25^{bright} (R3) indicated. Middle column: FOXP3/scurfin histograms for CD25- (R1), CD25^{int} (R2) and CD25^{bright} (R3) at baseline. Right column: as the middle histogram but at B cell repopulation.

in Fig. 7, the vast majority of FOXP3+ regulatory T cells reside within the CD25^{bright} gate. Thus, the increase of activated T cells upon repopulation did not lead to "contamination" of the CD25^{bright} population, these were still regulatory T cells. In our patient cohort we saw no indication of reduced numbers of regulatory T cells at baseline, upon B cell depletion and at repopulation these numbers were unaltered or even increased. Previous studies have suggested reduced frequencies of regulatory T cells in SLE patients [66,67] but these investigations did not include longitudinal studies nor was the focus on a homogenous patient group undergoing one treatment regime.

In contrast to the CD4+ T cell population, a high frequency of CD8+ T cells was found in the patients before treatment (Fig. 6E). However, due to the lymphopenia, the actual numbers of CD8+ T cells remained low even after a slight increase after rituximab treatment as compared to healthy subjects (data not shown). With the high level of activated CD4+ T cells in the patients, it became interesting to study the level of activation also in the CD8+ T cell compartment. Before treatment, only two of ten patients had a higher frequency of activated CD25+CD8+ T cells than the healthy controls (Fig. 6F). During recovery an increase close to three times occurred in all patients. Thus our data demonstrate important changes in several subpopulations of T cells. Further exploration of these changes will be needed in order to fully understand the effects of rituximab on the immune system.

Conclusions

In this study we have investigated the outcome of *in vivo* B cell depletion with rituximab on immune cells in a well-defined cohort of SLE patients. Our focus has been on antibody levels and lymphocytes, with special emphasis on T and B cell subsets longitudinally after therapy. With regards to antibodies, we saw a dramatic decrease in total IgM and IgE as well as in disease activity-associated IgG anti-dsDNA and anti-C1q antibodies. These data could indicate that certain antibodies are either produced by CD20 expressing B cell subsets or alternatively by short-lived plasma cells dependent on continuous renewal from the B cell pool. The major cellular effect after rituximab was a significant increase of both activated and regulatory T cells. Since naturally occurring regulatory T cells have previously been shown to be reduced in SLE, we confirmed by two complimentary methods that we were indeed studying regulatory T cells and that the baseline values were similar to those of healthy individuals. The observed increase could be due to two alternative scenarios (a) expansion or migration of thymic derived regulatory T cells or (b) induction of regulatory T cells from conventional T cells. The fact that rituximab induced disease remission in our patients makes it possible that the increase of both activated and regulatory T cell populations in the circulation is the result of decreased traffic to inflammatory sites. Importantly, regulatory T cells are capable of keeping the activated T cells in check, contributing to amelioration of the clinical disease. We attribute the phenotypic effects of the treatment mainly to the effects of B cell depletion and not as a major effect of cyclophosphamide, as all but two

patients were already at baseline treated with that drug. This conclusion is supported by an observed decrease in cell number and fewer activated T cells after cyclophosphamide treatment [68]. Also, the study by Anolik et al. describes a decrease in serum levels of anti-dsDNA antibodies, but unchanged IgG anti-tetanus antibody titers in SLE patients treated with rituximab only [24]. However, future studies are needed in order to determine to what degree cyclophosphamide influences the immunological effects observed in this study.

In conclusion, both the humoral and the cellular arms of the immune system, and both B and T cells are affected by rituximab treatment in patients with SLE.

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