Long-term repopulation of peripheral B-cell subsets after single and repeated rituximab infusions in patients with rheumatoid arthritis

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Abstract

Objective

B-cell depletion using rituximab (RTX) has proven efficacy in patients with RA. Long-term effects on the B-cell system after single and repeated treatments are sparse. Our aim was to study the effect of multiple courses of rituximab to evaluate its impact on repeated B-cell re-population capacity.

Methods

Two cohorts, altogether 20 patients with RA were included in an open label extension study with RTX. Cohort 1 received one cycle RTX and was followed for up to 7 years. In cohort 2 patients were studied under up to 5 cycles of RTX. Immunophenotyping was performed before therapy and during follow-up.

Results

After a single therapy with RTX (cohort 1) the frequency of pre-switch (MZ-like) B cells were significantly reduced during the follow-up of 7 years and absolute numbers slowly repopulated to nearly 50% of baseline value without numerical normalisation. The acquisition of mutations in Ig receptors of pre-switch (MZ-like) memory B cells was also significantly reduced 10 years after one course. In contrast, absolute numbers of (classical) post-switch B cells tended to normalise to baseline values after 7 years. Analysing B-cell repopulation capacities after multiple cycles revealed (cohort 2) a comparable repopulation pattern after each cycle with no substantial further impact on memory B cells.

Conclusion

A single therapy with RTX leads to long-term changes in the memory B-cell compartment particularly in pre-switch memory B cells. Multiple cycles of RTX show a comparable repopulation pattern after each cycle with no additional cumulative effect on memory B cells.

Key words

rituximab, rheumatoid arthritis, B cells

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Introduction

Rheumatoid arthritis is a systemic autoimmune disease characterised by chronic inflammation, synovitis, and progressive destruction of cartilage and bone in multiple joints. B cells have important roles in the pathogenesis of rheumatoid arthritis (1) including autoantibody production, acting as very sufficient antigen presenting cells, releasing proinflammatory cytokines and chemokines, which have essential effects on the development of tertiary lymphoid tissues (1-3). B-cell depletion with rituximab (RTX) is now well established in the treatment of rheumatoid arthritis (RA). Since its approval in 2006, a number of lessons could be learned, such as improved responsiveness to treatment by seropositive patients (4). In that context, approaches to individualise therapy and to identify cellular biomarkers have been reported (5-8). Clinical long-term data provided evidence for a repeated and lasting clinical efficacy with so far no further safety concerns (9).

In 2006, we and others demonstrated that B-cell repopulation after RTX follows a characteristic regeneration pattern (10, 11). In addition, we found profound changes in the memory B-cell compartment, which did not normalise to baseline values 2 years after a single therapy. Particularly pre-switch memory B cells showed a slow regeneration pattern in the short term (10) but long-term data on B-cell subsets after a single RTX course remained unclear.

RTX therapy usually requires regular retreatment in order to sustain clinical response (12), however, a number of patients show a long lasting response without the need for subsequent treatment cycles. Previously, we were able to show that 2 cycles with rituximab lead to a comparable regeneration pattern with no further impact on memory B-cell regeneration (13). Data on the status of serum immunoglobulin levels show that they stay mainly in the normal range after repeated cycles (11). Only a small number of patients develop immunoglobulin G levels below the lower limit of normal after receiving up to 5 cycles of rituximab (3.5–22%) (9, 14), whereas low IgM levels are found in 22–38.8% of patients after repeated cycles (9, 21).

It has been hypothesised that repeated cycles of rituximab exert cumulative effects on the memory B-cell compartment and interfere indirectly with plasmablast/plasma cell dynamics (15). To date, cellular data describing the repopulation capacity of the B-cell compartment after multiple courses of rituximab are lacking. Retreatment with rituximab is usually administered at a time point when the peripheral B-cell compartment has not yet been restored. Therefore, we studied the effect of multiple courses of RTX to evaluate its impact on repeated B-cell re-population capacity.

Patients and methods

Patients characteristics and study design

Altogether 20 patients were included in an open label trial with RTX initiated in October 2000. All patients met the American College of Rheumatology criteria for RA. Informed consent was obtained from all patients before entering the study in accordance with the protocol approved by the ethics committee of the University of Würzburg, Germany.

Cohort 1: single therapy

In cohort 1 seven RA patients were included. These patients received one cycle of RTX (4 patients received 1g RTX 2 weeks apart and the other 3 four weekly infusions of RTX at a dose of 375 mg/m^2). All but one patient stayed on concomitant methotrexate. Of these 7 patients, all but one was positive for RF and ACPA antibodies. Patients were followed up yearly, and immunephenotyping of peripheral B-cell subsets was performed. Baseline and week 24 clinical and laboratory data after the single RTX cycle are provided in Table I. Due to the great variety of follow-up treatments subgroup analysis based on clinical data was not appropriate. Four patients were not retreated due to lack of response. Three patients were not retreated due to lack of licency since they were all given RTX as part of the open label trial. After the single RTX course patients were treated with different biological agents (Table II). Altogether the observation time in median was 7.5 years (range 7–11.4 years).

Table I. Clinical and laboratory data of 7 patients with a single therapy with RTX.

Data are expressed as median and range

	Baseline	W24
Age (years)	48 (33-62)	
% Female	84	
Disease duration (years)	14 (1-19)	
DAS28 score	5.5 (4.6-6.4)	4.9 (3.4-6.3)
RF (< 10 U/ml)	188 (42-391)	103 (33-341)
IgG (662-1271 U/ml)	1209 (865-2000)	1220 (842-1490)
IgA (85-499 U/ml)	245 (109-333)	2112 (136-328)
IgM (53-209 U/ml)	202 (73-316)	147 (70-240)
ESR (<15 mm/1h)	32 (12-84)	29 (13-46)
CRP (< 0.5 mg/dl)	1.2 (0.5-14.3)	1.7 (0.4-14.9)

Cohort 2: repeated treatment

In cohort 2, thirteen patients with RA received repeated treatments of 1g RTX 2 weeks apart according to the physicans discretion and the disease activity. All patients stayed on concomitant methotrexate. In this cohort, 9/13 were ever positive for both RF and ACPA, in the other 4 patients one were RF positive and all 4 were negative for ACPA. All patients received 3 cycles of RTX, 8 patients 4 cycles and 6 patients received 5 cycles. Peripheral blood samples for immunephenotyping were taken at baseline, early in repopulation (with CD19+ B cells >1%) and directly prior to the start of subsequent therapy. The clinical and laboratory data are provided in Table III. As the follow-up of the B-cell compartment was the main focus of the study, selected other clinical and laboratory data are only shown when appropriate. The median (range) time span between the cycles were as follows: 17.7 months (9.4–83.7) between first and second treatment, 21.9 months (6.3–59) between second and third treatment, 14.4 months (9.8–33) between third and fourth treatment and 13.6 months (8.6–39.1) between fourth and fifth treatment, respectively.

Phenotypic B-cell analysis and Ig mutational rate

Peripheral blood was collected from patients and was prepared as described previously (10). Immunephenotyping was done by using four colour staining using a FACSCalibur (Becton Dickinson, San Jose, CA) with the following mAb: CD19 (APC), CD38

Table II. Overview of follow-up therapies in 7 patients with a single rituximab therapy.

	1 year	2 year	3 year	4 year	5 year	6 year	7 year
Pat. 1	Adalimumab/ MTX	Abatacept/ MTX	Etanercept/ Leflunomid	Tofacitinib/ MTX	Tocilizumab/ MTX	Tocilizumab/ MTX	Tocilizumab/ MTX
Pat. 2	Adalimumab	Etanercept	Etanercept	Etanercept	Etanercept	Etanercept	Etanercept
Pat. 3	Infliximab/ MTX	Infliximab/ MTX	Etanercept/ MTX	Etanercept/ MTX	Tocilizumab/ MTX	Tocilizumab/ MTX	Tocilizumab/ MTX
Pat. 4	MTX	MTX/ Leflunomid	MTX/ Leflunomid	MTX/ Leflunomid	MTX/ Leflunomid	Adalimumab/ MTX	Abatacept/ MTX
Pat. 5	MTX	Anakinra/ MTX	Anakinra/ MTX	Adalimumab/ MTX	Adalimumab/ MTX	Adalimumab/ MTX	Adalimumab/ MTX
Pat. 6	MTX	Anakinra/ MTX	Infliximab/ MTX	Infliximab/ MTX	Tocilizumab/ MTX	Tocilizumab/ MTX	Certolizumab/ MTX
Pat. 7	Sulfasalazin	Etanercept	no therapy	no therapy	no therapy	Tocilizumab	Etanercept

Table III. Clinical and laboratory data of 13 patients with repeated treatments of RTX.

Data are expressed as median and range. 10/13 patients were positive for RF

	Baseline	W24 after 1st th	W24 after 2nd th	W24 after 3rd th	W24 after 4th th	W24 after 5th th
Age (years)	49.5 (27-77)					
% Female	70					
DD (years)*	6 (3-26)					
DAS28 score	6 (5.1-8)	4.6 (2.1-5.4)	2.5 (1.8-5.2)	3.2 (1.7-5.2)	3.1 (1.2-5.3)	3.4 (2.8-4.2)
RF (<10U/ml)	206 (133-378)	63 (37-217)	32 (16-77)	29 (10-47)	22 (14-52)	21 (17-26)
IgG (662-1271U/ml)	1180 (742-1840)	1080 (823-1150)	1022 (768-1310)	998 (510-1219)	1071 (786-1221)	1057 (707-1386)
IgA (85-499U/ml)	169 (105-404)	159 (135-517)	244 (131-385)	154 (72-351)	212 (119-373)	245 (118-304)
IgM (53-209U/ml)	168 (68-394)	134 (42-298)	104 (29-135)	53 (20-114)	93 (29-118)	56 (31-170)
ESR (<15mm/1h)	29 (19-83)	14 (7-67)	14 (5-32)	18 (9-39)	10 (5-56)	8 (8-11)
CRP (<0.5mg/dl)	2.6 (1-6.2)	0.9 (0.03-3.3)	0.6 (0.01-3.5)	0.7 (0.03-3.4)	0.3 (0.04-2)	0.3 (0.08-0.8)

*DD: disease duration; th: therapy.

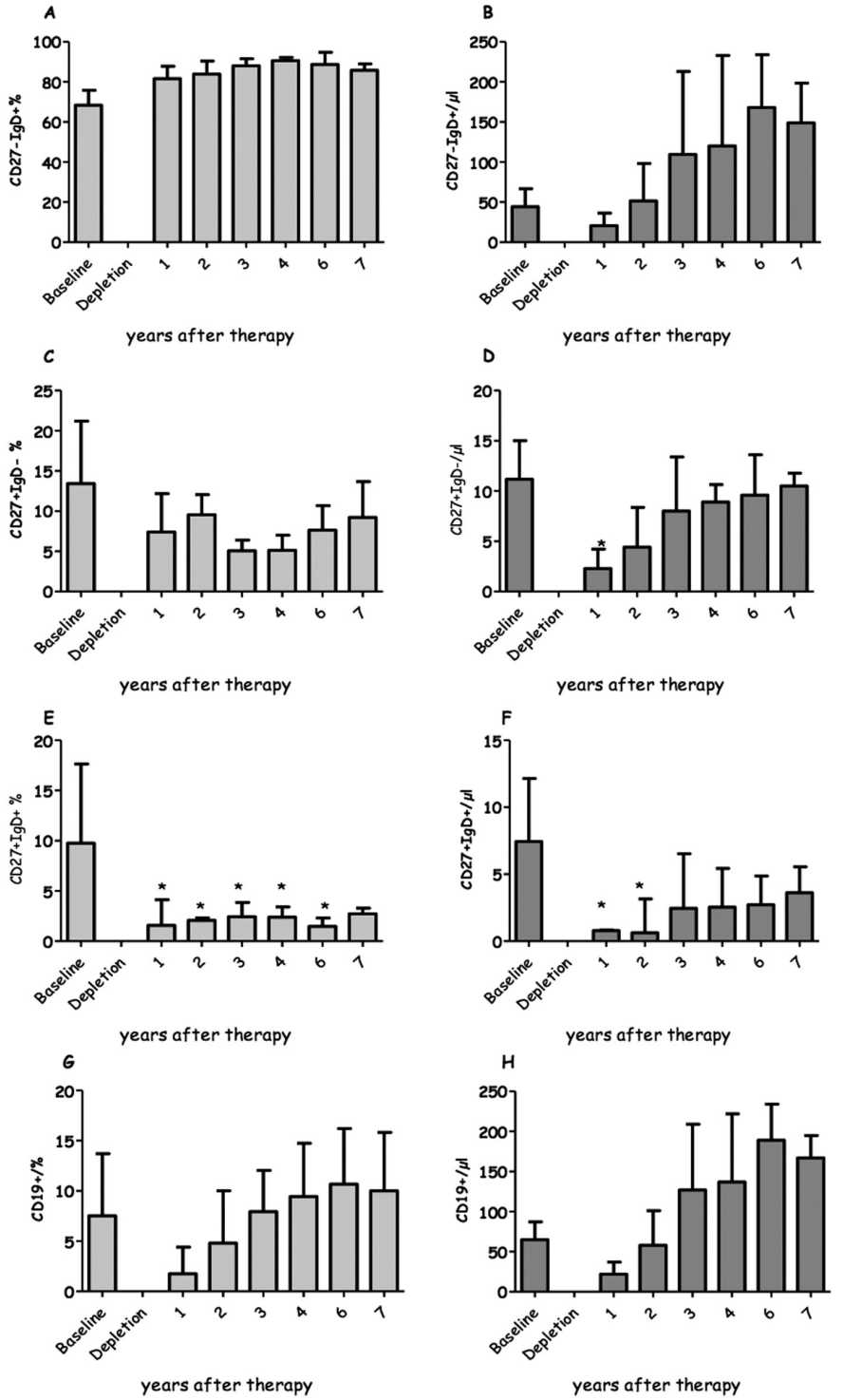

Fig. 1. Long-term course of the peripheral B-cell compartment up to 7 years after a single cycle of rituximab. Relative numbers of naïve CD27-/IgD+ B cells normalised after the first year of rituximab therapy (**A**). Absolute numbers normalised 2 years after treatment. (**B**). The memory B-cell compartment showed a delayed repopulation with incomplete normalisation after 7 years (**C-F**).
Course of relative and absolute numbers of CD19+ B cells (**G/H**). Analysis of the 5th year is incomplete. Values are median and interquartile range, *p<0.05.

(PerCPCy5.5), CD10 (PE), CD27 (PE) and anti human IgD (FITC), isotype control antibodies used were: Mouse G1/G2a (FITC/PE), Mouse IgG1 (PerCPCy5.5), Mouse IgG1 (APC). All antibodies were from Becton Dickinson (Heidelberg, Germany). Frequencies of CD19+ cells were calculated using CellQuest software (Becton Dickinson).

Statistical analysis

All statistical analysis were performed using Graph pad prism. The non-parametric Mann-Whitney test was used to compare baseline values with yearly values of patients with a single therapy. Immunephenotypic data were expressed as median and interquartile range. p<0.05 was considered to be statistically significant. Clinical and laboratory data were expressed as median and range.

Results

Long-term repopulation of the B-cell compartment after a single therapy cycle with RTX (cohort 1):

• *Naïve B-cell compartment after a single therapy with RTX*

The long-term follow up of the B-cell compartment in 7 patients treated with one course of RTX showed a number of interesting findings. At baseline the relative and absolute numbers of overall CD19+ B cells were 7.5% (5.2–13.7) and 65/µl (44.8–143.6), respectively (Fig. 1 G/H). Three years after a single therapy with RTX these numbers were normalised to 7.9% (6.4–12.0) and 127/µl (51–209), respectively. The frequency of naïve CD27-/IgD+ B cells reached baseline values within the first year (Fig. 1A) and stayed elevated until 7 years after therapy. Absolute numbers of naïve B cells reached baseline values during the second year after therapy (Fig. 1B) (baseline: 44.3/µl (17.8–66.8), second year: 51.5/µl (21.7–98.1)) and expanded in the following 7 years after therapy with RTX compared to baseline levels (7 year: 148.8/µl (63.7–198.5)).

• *Memory B-cell compartment after a single therapy with RTX*

Notable, pre- (MZ-like) and post-switch (classical) memory B cells showed a different repopulation kinetic. Relative numbers of CD27+/IgD- post-switch memory B cells showed an increase to 70% of baseline value in the first 2 years after therapy with RTX with no further elevation in the follow-up of 7 years (Fig. 1C). However, absolute numbers of post-switch B cells increased continuously after B-cell depletion and reached nearly

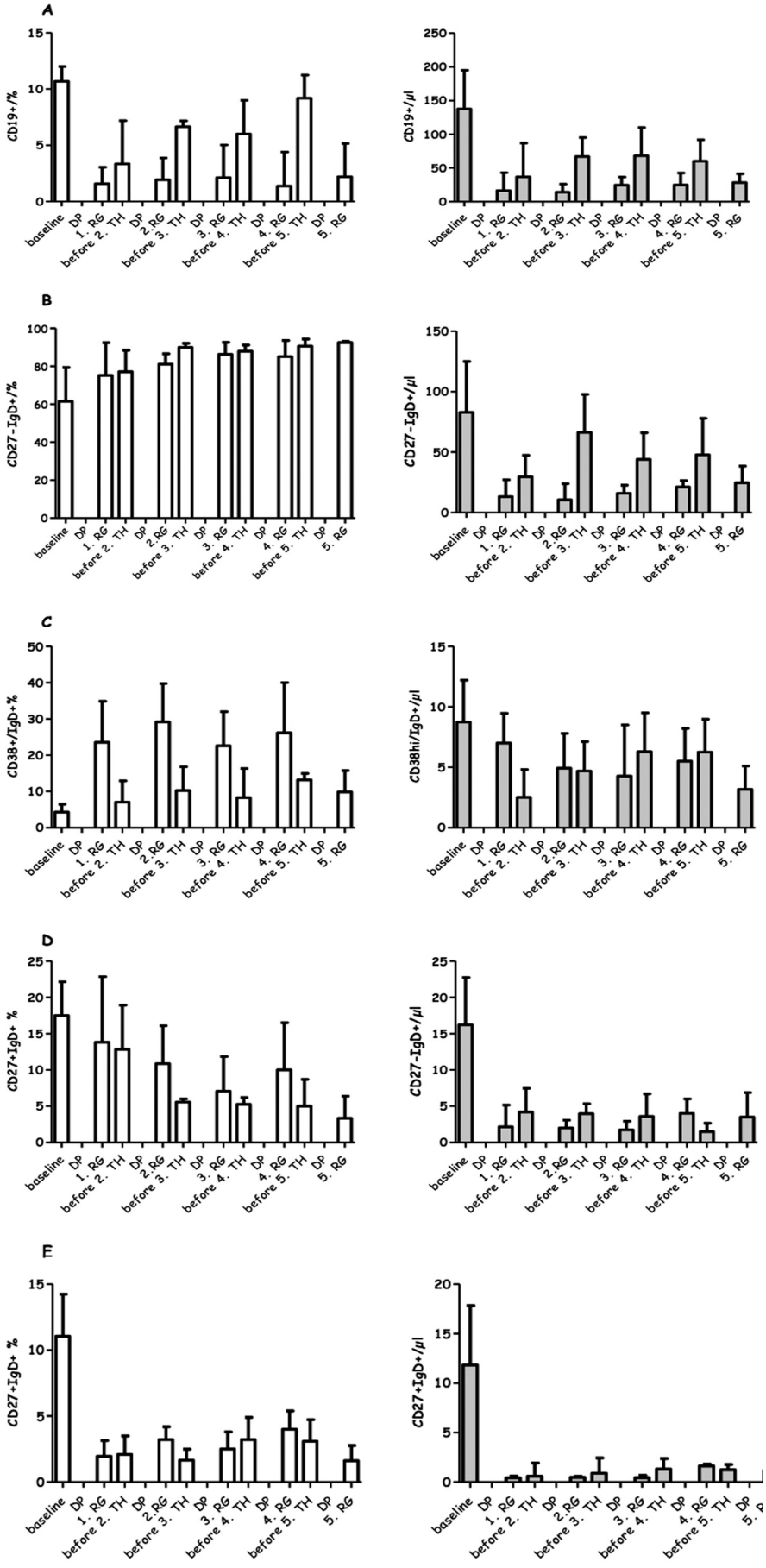

Fig. 2. Time course of different peripheral B-cell subsets after 5 repeating cycles with rituximab. The time span between the first regeneration and the second treatment was in median 9.8 months (range 2.4-74.6), for the second regeneration and the third treatment 18.2 months (2.0-48.3), for the third regeneration and the fourth treatment 6.2 months (2.2-22.1) and for the fourth regeneration and the fifth treatment 7.2 months (2.2-31.4), respectively. 13 patients received 3 cycles, 8 patients 4 cycles and 6 patients 5 cycles of rituximab. Stated are baseline values, time points of early repopulation and last time point before the next therapy.
DP: Depletion, RG: Regeneration, TH: Therapy. Data are expressed as median and interquartile range.

baseline values (11.2/µl, 3.8–15) after 7 years (10.5/µl, 9.5–11.8) (Fig. 1D). The frequency of CD27+/ IgD+ pre-switch (MZ-like) memory B cells was significantly reduced during the observation period of 7 years (baseline value: 9.7% (5.2–17.7), increase 3rd year: 2.4% (1.8–3.8) and 2.7% (1.1–3.3) in the 7th year, $p<0.05$). Absolute numbers of pre-switch B cells were significantly reduced during the first 2 years after therapy with RTX (Fig. 1F, $p<0.05$). Thereafter this subset slowly increased to nearly 50% of baseline value in the 7th year after therapy with rituximab but showed no sufficient repopulation (baseline value: 7.4/µl, 4.5–12.2, 7th year: 3.6/µl, 2.0–5.5).

Repopulation capacity of the peripheral B-cell compartment after 5 repeated cycles of RTX (cohort 2):

• *Naïve B-cell subsets after repeated RTX cycles*

Overall CD19+ B cells always showed a similar repopulation pattern after each cycle without a tendency to regenerate at lower numbers after repeated cycles (Fig. 2A). As reported before (12), the early repopulation phase was dominated by high relative numbers of CD38hi/IgD+/CD10+ transitional B cells, which subsequently decreased in the later regeneration phase (Fig. 2C). Similarly, absolute numbers of these cells repopulated comparable to baseline values. Relative numbers of naïve CD27-/IgD+ B cells showed a fast repopulation after immediate peripheral B-cell depletion (Fig. 2B) and repopulated at increased relative numbers after repeated cycles of therapy.

• *Time course of the memory B-cell compartment after repeated treatments*
The memory B-cell compartment however, showed a different repopulation kinetic. The frequency of post-switch (classical) CD27+/IgD- B cells showed increased numbers early in the repopulation phase which was not as pronounced in subsequent cycles. This was caused by an early emergence of recirculating CD38hi/IgD- plasmablasts particularly during the first courses of RTX (4). Absolute numbers of post-switch (classical) memory B cells increased to a maximum of one third after each cycle compared to baseline values and stayed in this range also for up to 5 cycles of RTX. Of note, pre-switch (MZ-like) CD27+/IgD+ B cells also remained at low frequencies of about one fourth of baseline values after the first RTX therapy but showed no further decrease even after 5 cycles in relative and absolute numbers (Fig. 2E).

Discussion

In this study we describe long-term effects of single and repeated courses of RTX on the peripheral B-cell compartment. The analysis over a time period of 7 years revealed ongoing profound changes even after a single course of RTX. The naïve B-cell compartment showed good repopulation in the short term with further expansion of relative and absolute numbers over longer periods of time. Interestingly, the memory B-cell compartment remained numerically reduced 7 years even after a single therapy with RTX but provided differences between the two cohorts.

While relative numbers of post-switch memory (classical) B-cell subsets showed a repopulation to approximately 70% of baseline values, absolute numbers tended to normalise during the observation period of 7 years. However pre-switch (MZ-like) memory B cells showed a different repopulation kinetic. The frequency of this subset was still significantly reduced after 7 years, whereas absolute numbers showed a reduced repopulation to nearly 50% of baseline value but without a numerically normalisation even though the overall CD19 count increased. It has been previously shown that also the mutational frequency in single Ig B-cell receptors was significantly reduced at 6 years after RTX (16). We now extend these findings and identified that even 10 years after a single RTX therapy reduced mutations occur in repopulated pre-switch (MZ-like) memory B cells (baseline mutation per sequence 9.89±0.6 *vs*. 10 years: 7.7±0.69, p=0.04) (data not published). The impaired repopulation capacity of pre-switch (MZ-like) memory B cells was unexpected, since data of infants show a gradually maturation of this population reaching adult values by 2–3 years of age (17). A recently published study also reported a significant reduction of CD27+ cells in bone marrow and in the circulation of 13 patients at one time point (mean 22 months, 6–61 months) after treatment with RTX (18) affecting both, pre- and post-switch memory B cells. This raises questions of additive factors required for an unimpaired development of pre-switch (MZ-like) memory B cells which may be compromised by RTX. Factors like cytokine milieu, microenvironment and the interaction with other cells may be responsible for the current observations. Alternatively pre-switch (MZ-like) memory B cells may repopulate from a so far not clearly described "progenitor cell" which is targeted by RTX and shows a reduced renewal potential in adults. In splenectomised patients a protracted reduction of pre-switch memory B-cell populations is observed suggesting that the spleen plays an important role in the development of these cells (17, 19) as known for MZ B cells. These questions require further investigations. It remains unclear what effect these observations may have on the patients' health. Apart from a reduced response to polysaccharide vaccines (20) no elevated infection rates in particular for encapsulated bacteria have been reported so far (9).

Our data on long-term B-cell reconstitution may have been influenced by medically required further immunosuppressive therapies. Particularly IL-6 inhibition with tocilizumab has been described to influence the memory B-cell compartment. 4/7 of our patients have been treated at some time after the single RTX course with tocilizumab (3 patients at the 5th year and 1 patient at the 6th year after RTX). However, these patients showed a comparable repopulation pattern in which post-switch B cells gradually further increased and pre-switch B cells showed no increasing reduction of absolute numbers after tocilizumab. Therefore, a clear influence of tocilizumab could not be identified under these conditions.

In the second part of our study, we investigated the repopulation capacity of the peripheral B-cell compartment during 5 repeated cycles of rituximab. Overall, CD19+ B cells showed a comparable repopulation kinetic after each cycle. The naïve B-cell compartment repopulated with a trend for increased numbers after each cycle and the memory B-cell repopulation was comparable to the described repopulation after one course. Memory B-cell repopulation after repeated cycles is of special interest in the context of the delayed repopulation and still incomplete peripheral numbers after the first course. It may be hypothesised that repeated cycles of rituximab exert cumulative effects on the memory B-cell compartment especially on classical CD27+IgD- B cells and interfere with plasmablast/ plasma cell dynamics which eventually may lead to exhausting B-cell memory with direct influences on infections rates or on protective vaccination titres. Recently the influence on immunoglobulin levels after repeated courses of rituximab were reported (21). It could be shown, that particularly patients with lower baseline immunoglobulin levels before initiating rituximab tended to develop persistent IgM and IgG hypogammaglobulinaemia after repeated cycles. After the first and second cycle more patients developed low IgG levels than after the third and forth cycle. In contrast, low IgM levels were seen in more patients with progressive cycles arguing for a failure of naïve or pre-switch memory B cells to differentiate into plasma cells (20). In our study no patient had low IgM or IgG levels at baseline but one patient developed IgG and IgM hypogammaglobulinaemia after the third cycle. In our study we did not observe progressive reductions of pre and post-switch

memory B cells after repeated cycles in our patient group nor in the single hypogammaglobulinaemiac patient.

In conclusion, RTX leads to substantial changes in the memory B-cell compartment, particularly noticeable for pre-switch (MZ-like) memory B cells, which are still detectable 7 years after a single RTX therapy. Repeated cycles show a comparable B-cell repopulation pattern with repeating recovery of similar numbers of memory B cells after each RTX cycle but apparently defines a RA cohort that requires continuous RTX depletion to control classical memory B cells while for another cohort this seems to less important. Overall, the two different cohorts suggest that the response to RTX as well as the kinetics of classical memory B cells permit the conclusion that different immunopathogenic drivers are effective in RA.

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