

# Rituximab Therapy Leads to Reduced Imprints of Receptor Revision in Immunoglobulin $\kappa$ and $\lambda$ Light Chains

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**ABSTRACT. Objective.** Transient B cell depletion by rituximab (RTX) has become a specific treatment of rheumatoid arthritis (RA). Although phenotypic repopulation kinetics of B cell subsets are well documented, precise molecular analyses of the reconstituting immunoglobulin (Ig) genes encoding the B cell receptor in RA are sparse.

**Methods.** A total of 708 individual CD19+CD27+ (memory) and CD19+CD27- (naive) B cells from 2 patients with RA were analyzed at baseline and 7 months after RTX at B cell repopulation. Ig light chain variable kappa ( $V\kappa$ ) and lambda ( $V\lambda$ ) light chain gene rearrangements were amplified, sequenced, and analyzed with a focus on receptor revision.

**Results.** The naive as well as the memory repertoire repopulated polyclonally with diverse use of variable light chain gene families and minigenes. During the reconstitution phase, B cells used significantly fewer J $\kappa$  distal V $\kappa$  genes ( $p = 0.0006$ ), with a higher frequency of somatic hypermutation of rearrangements employing J $\kappa$ 5 compared to baseline in memory B cells. The use of V $\lambda$  rearrangements in regenerating B cells was also biased toward use of V $\lambda$  genes of the proximal cassette. In general, reemerging CD27+ Ig light chain genes were substantially more highly mutated than before RTX therapy ( $p < 0.0001$ , baseline vs during reconstitution).

**Conclusion.** Our data indicate that RTX therapy leads to generation of distinct V $\kappa$ /J $\kappa$  and V $\lambda$ /J $\lambda$  gene repertoires consistent with replenishment of antigen-experienced B cells by germinal centers. At baseline, the imprints of receptor revision appeared to be more striking, which indicates that receptor revision is active in patients with RA and can be reduced by RTX. (First Release April 15 2012; J Rheumatol 2012;39:1130-8; doi:10.3899/jrheum.111513)

## Key Indexing Terms:

RHEUMATOID ARTHRITIS IMMUNOGLOBULIN LIGHT CHAINS RECEPTOR REVISION

Rheumatoid arthritis (RA) is an immune-mediated systemic inflammatory disorder manifested by aggressive synovitis that leads to progressive cartilage degradation and bone erosion, which causes pain, joint deformity, and functional dis-

ability<sup>1,2</sup>. Whereas different cell types play pathogenic roles in RA, B cells have drawn particular attention<sup>3</sup>. B cells do not merely secrete autoantibodies including rheumatoid factor (RF) and antibodies against citrullinated peptides, but are also involved in secretion of proinflammatory cytokines, activating autoreactive T cells, and presenting and processing autoantigens, leading to infiltration of end organs<sup>4</sup>. B cells are able to rearrange specific antigenic receptors by receptor-editing and lack of central and/or peripheral tolerance contributes to their abnormal activation and proliferation in autoimmune diseases<sup>5,6</sup>. The importance of B cells in the pathophysiology of RA has been emphasized by therapeutic approaches using the anti-CD20 monoclonal antibody rituximab (RTX). RTX results in prolonged alleviation of clinical symptoms and inflammation and reduced erosive joint destruction<sup>7</sup>. We and other investigators have reported regeneration kinetics from B cell depletion studies indicating that peripheral B cells repopulate between 6 months and 10 months after depletion with a majority of newly emerging immature B cells<sup>8,9</sup>.

The B cell repertoire is shaped by a complex set of genes that are modulated through different rearrangements, somat-

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ic hypermutation, and receptor revision. All these processes are involved in producing unique and diverse immunoglobulin (Ig) receptors and are highly regulated during ontogeny, development, and response to antigen. Selection and activation of autoreactive B cells in healthy individuals is avoided by a tolerance mechanism and the vast majority of naive autoreactive B cells are deleted through apoptosis in their premature stages<sup>10,11</sup>.

B cell antigen receptor revision involves secondary Ig gene rearrangement. Ig-transgenic mouse model studies identified receptor revision events as a relatively rare process capable of rescuing a small fraction of autoreactive B cells<sup>12</sup>. The IgV repertoire is shaped by a variety of selective and molecular events. Previous studies indicated the existence of disrupted receptor revision in RA<sup>13</sup>. However, no data are available analyzing the light chain repertoire under RTX, which might follow different expression profiles and could be exposed to distinct selection pressures.

We analyzed the molecular aspects of B cell receptor after transient B cell depletion in patients with RA, with a focus on modulation of B cell immunoglobulin V $\kappa$  and V $\lambda$  light chain gene rearrangements in CD27 $-$  naive and CD27 $+$  memory B cells. In particular, we analyzed the mutational pattern and Ig gene repertoire before B cell depletion and during the B cell regeneration phase (7 months) after a single dose of RTX.

## MATERIALS AND METHODS

**Patients.** Informed consent was obtained from the patients according to a protocol approved by the ethics committee of the University of Würzburg and according to the Declaration of Helsinki. Patient 1 (female, 47 years old) and Patient 2 (female, 35 years old) had active RA.

Both patients were RF-positive and were diagnosed according to the American College of Rheumatology revised criteria for RA<sup>14</sup>. They were nonresponders to standard disease-modifying antirheumatic drugs. Patients were receiving a stable dose of methotrexate before receiving 2 treatments of 1000 mg RTX, 2 weeks apart. Disease activity was determined by measuring the 28-joint Disease Activity Score, erythrocyte sedimentation rate, and C-reactive protein levels. Further disease variables are shown in Table 1. Both patients showed a clinical response during the study. Patient 2 experienced increasing disease activity at the second timepoint of analysis.

**Single cell sorting.** For separation of peripheral mononuclear cells, a blood sample suspension was centrifuged on a Ficoll-paque gradient (GE Healthcare, Munich, Germany) with subsequent washing steps in phosphate buffered saline (PBS). A single B cell sorting technique was per-

formed as described<sup>15,16</sup>. In brief, B cells were stained with fluorescence-labeled monoclonal antibodies against B cell surface markers: CD19-APC and CD27-PE. Cell incubation with antibodies was performed at 4°C for 15 min in PBS/0.5% bovine serum albumin (BSA). Individual B cells were sorted into 96-well plates containing lysis buffer using a FACS DiVA process (Beckton Dickinson). Lysis buffer consisted of DTT (Roche Diagnostics, Mannheim, Germany), RNasin (Promega), Oligo dT(15) primer (Promega), BSA (Sigma-Aldrich), Triton 10% (EMD), and molecular grade H<sub>2</sub>O.

**cDNA synthesis.** A mixture containing nucleotides, reverse transcriptase, and RT-polymerase chain reaction buffer from the Titan One Tube RT-PCR System (Roche) was added into each well of a 96-well plate containing sorted B cells. cDNA was synthesized by incubation at 50°C for 1 h.

**Nested polymerase chain reaction (PCR).** V $\kappa$  (families 1–6) and V $\lambda$  (families 1–10) were amplified by nested PCR using family-specific primers. Reaction mixture, primers, and cycling conditions were as described<sup>17,18</sup>. PCR products were separated by electrophoresis on a 1.5% agarose gel. The products were purified further with a MinElute Gel Extraction kit (Qiagen, Hilden, Germany).

**Sequencing and analysis.** Gel-extracted V $\kappa$  and V $\lambda$  family products were further amplified using a BigDye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer Applied Biosystems, Weiterstadt, Germany) and 5' internal primers of each family followed by sequencing in an automated genetic analyzer, the ABI Prism 310 (Applied Biosystems). Sequence analysis was performed using the online program JOIN-SOLVER<sup>®19</sup> (US National Institutes of Health, National Institute of Arthritis and Musculoskeletal and Skin Diseases) and productively rearranged sequences were matched to the closest germline configuration in the ImMunoGeneTics information system database. Out of frame and nonproductive sequences with a stop codon were excluded from the analysis.

**Statistical analysis.** Statistical analyses were performed using GraphPad Prism 3.03 (GraphPad Software, San Diego, CA, USA). Frequencies of V $\kappa$  and V $\lambda$  families as well as J $\kappa$  and J $\lambda$  gene usage were compared using 2-tailed Fisher's exact test. Chi-square test was used to compare the mutational frequencies, RGYW/WRCY mutations, and R/S mutations, while CDR3 lengths were compared by nonparametric Mann-Whitney U test. P values < 0.05 were considered statistically significant.

## RESULTS

**Distribution of V $\kappa$  and V $\lambda$  family repertoire.** In order to identify potential therapy-related molecular modifications in circulating B cells, we studied the Ig light chain gene usage of 2 RA patients in detail. CD19+CD27 $+$  memory and CD19+CD27 $-$  naive B cells from both patients used light chain gene rearrangements in similar proportions in their productive repertoires, with no significant differences. Therefore, we grouped the Ig sequences from both patients

**Table 1.** Laboratory and clinical variables of the patients treated with rituximab. B cell subsets were analyzed by flow cytometry. Results are shown as percentage of CD19 $+$  lymphocytes.

	SJC	TJC	DAS28	CRP, mg/dl	ESR, mm/h	CD27+IgD+, %	CD27+IgD-, %	CD27++ CD38+++ , %	CD27-IgD+, %
<b>Patient 1</b>									
Baseline	9	10	4.84	0.56	12	15.5	14.0	2.5	67.5
After RTX	6	5	3.6	0.47	8	2.5	11.0	3.5	81.0
<b>Patient 2</b>									
Baseline	15	11	5.80	8.71	33	12.0	16.5	2.7	63.0
After RTX	12	7	5.51	5.42	38	1.0	13.0	4.7	76.0

SJC: swollen joint count; TJC: tender joint count; DAS: Disease Activity Score; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate.

and examined the changes in the light chain repertoire in terms of variable (V) and joining (J) gene usage, CDR3 length, and mutational imprints.

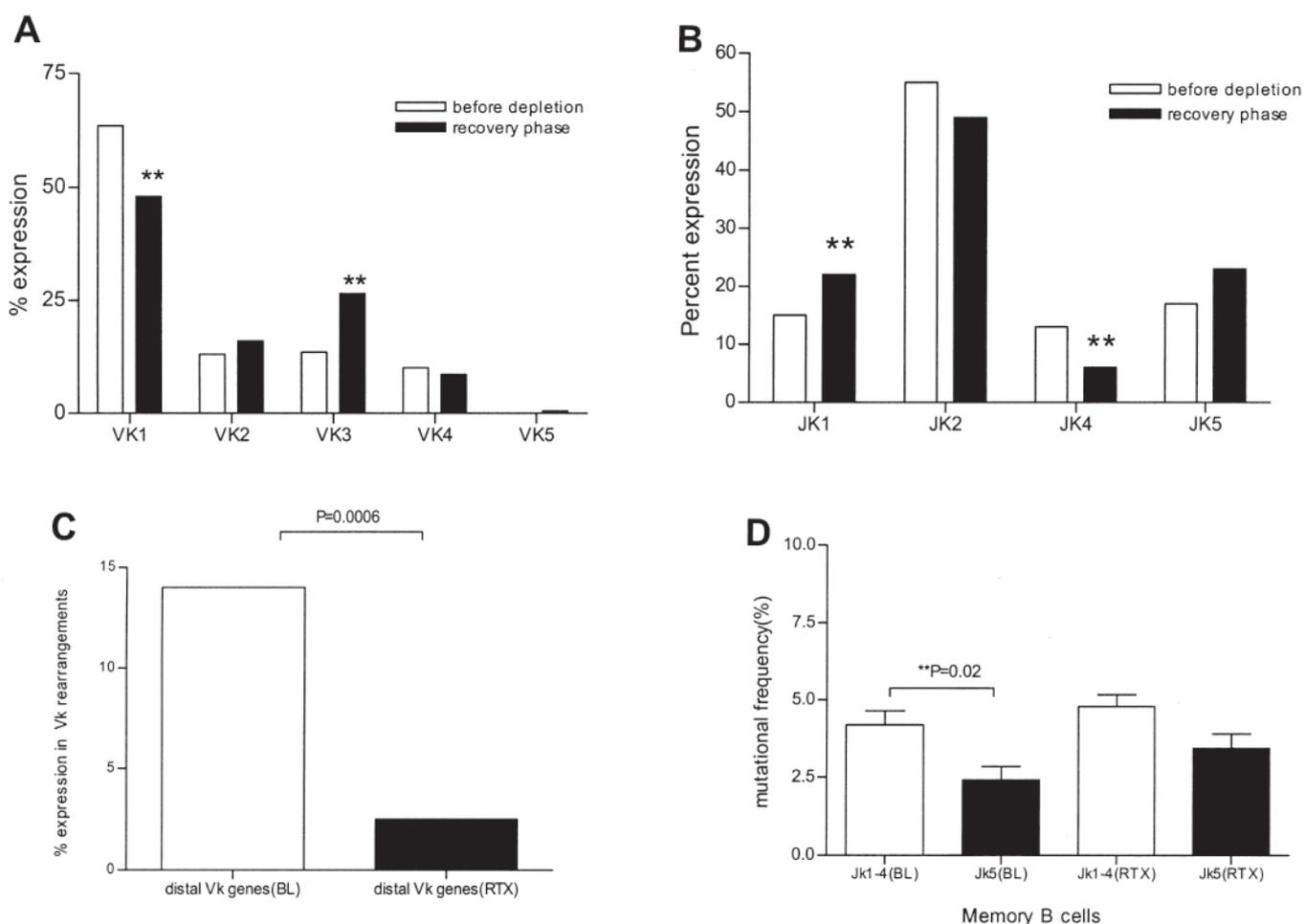
Before depletion, V $\kappa$ 1 family members were significantly overexpressed, followed by V $\kappa$ 2 and V $\kappa$ 3 family members. V $\kappa$ 4 family genes were observed at lower frequencies, whereas no V $\kappa$ 5 or V $\kappa$ 6 family members were found. During repletion after RTX, a number of changes of V family gene usage were observed. The frequency of occurrence of V $\kappa$ 1 family members decreased (63.5% vs 48%;  $p < 0.05$ ), while occurrence of V $\kappa$ 3 genes increased (13.5% vs 26.5%;  $p < 0.05$ ; Figure 1A). The frequencies of V $\kappa$ 2 and V $\kappa$ 4 family members before and after RTX were not statistically significantly different.

V $\lambda$  light chain analysis showed that V $\lambda$ 1, 2, and 3 family members were predominantly employed by the expressed repertoire before and after depletion with V $\lambda$ 2 genes, fol-

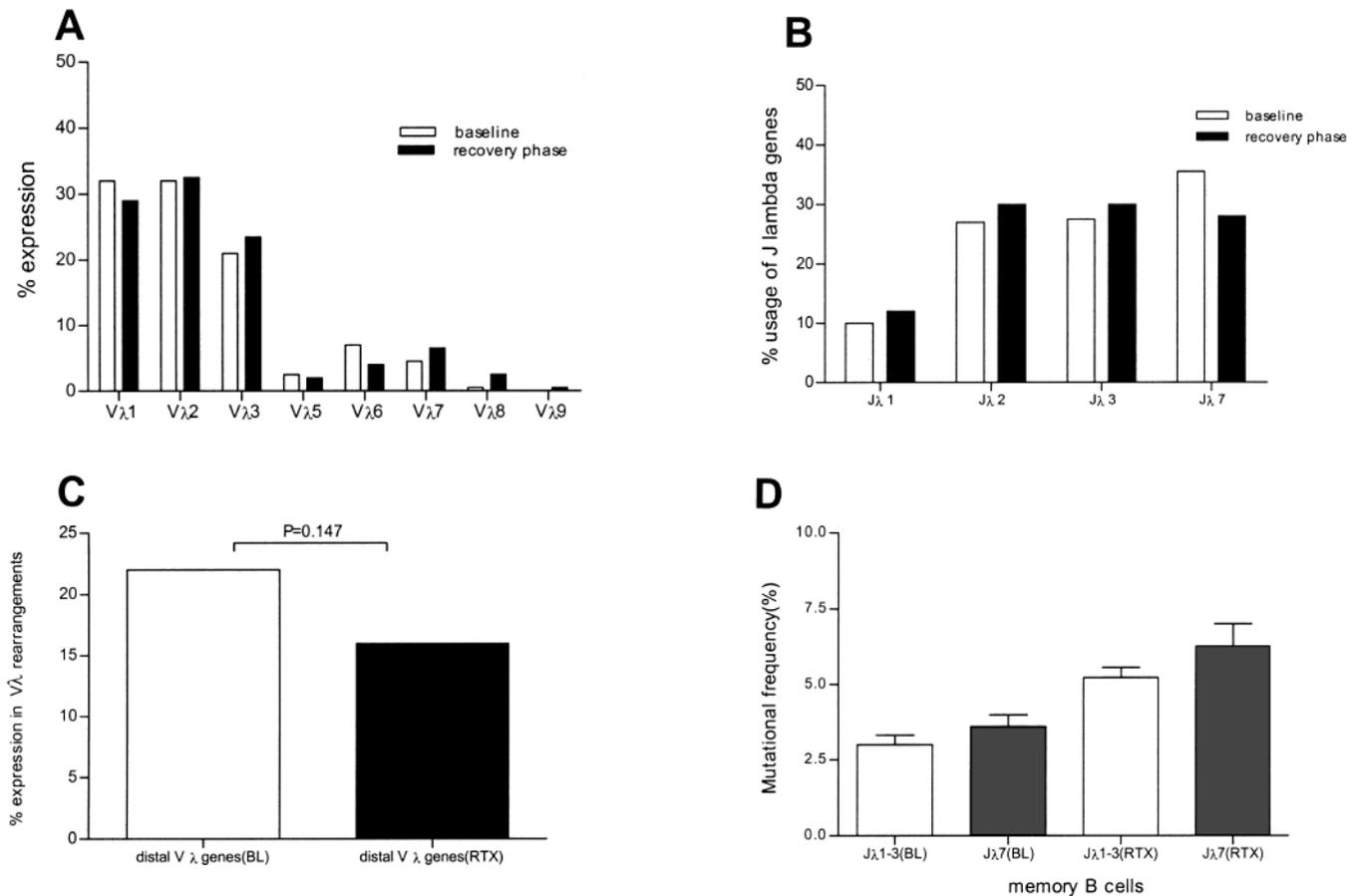
lowed by V $\lambda$ 1 predominantly used genes before and after therapy. V $\lambda$ 6, 7, 8, and 9 family members were observed at very low percentages. Usage of V $\lambda$  families remained mainly stable between baseline and the followup after B cell depletion (Figure 2A).

Of note, the relation between V $\kappa$  and V $\lambda$  gene usage was comparable between the 2 timepoints (52% V $\kappa$  vs 50% during regeneration; 48% V $\lambda$  vs 50% during regeneration), excluding the possibility that RTX may have led to different usage of the 2 light chain gene repertoires.

*Usage of Ig V $\kappa$  and V $\lambda$  minigenes by the CD19+/CD27- naive B cell repertoire.* Analysis of individual V $\kappa$  minigene usage revealed that a wide range of V $\kappa$  genes were employed by CD27- B cells and showed a number of changes after therapy (Figure 3). Certain genes, such as V $\kappa$ 1-5.03 and V $\kappa$ 1-39.01, were used very often. Moreover, minigenes like V $\kappa$ 1-6.01, 1D-8.01, 1D-13.01, 1-37.01, and



**Figure 1.** Use of V $\kappa$  and J $\kappa$  minigenes before B cell depletion by rituximab and during recovery phase. A. V $\kappa$ 1 use was higher among all families and decreased significantly after therapy, while V $\kappa$ 3 gene usage was significantly increased during recovery phase. B. Significant changes in J $\kappa$ 1 and 4 minigene usage (\*\* $p < 0.05$ ). C. Distribution of individual V $\kappa$ s gene rearrangements indicate that J $\kappa$  distal V $\kappa$  genes used at baseline were altered significantly by rituximab (RTX) therapy. At baseline, 14% of rearrangements used J $\kappa$  distal V $\kappa$  genes, while during RTX therapy only 2.5% of rearrangements used J $\kappa$  distal V $\kappa$  genes ( $p = 0.0006$ ), while the remainder of the rearrangements used J $\kappa$  proximal V $\kappa$  genes. D. Comparison of mutational frequencies between V $\kappa$  gene rearrangements in memory B cells using J $\kappa$ 5 and those using J $\kappa$ 1-4 before (BL, baseline) and after (RTX) therapy. \*\*A significant difference in mutational frequencies between J $\kappa$ 1-4 and J $\kappa$ 5 using V $\kappa$  rearrangements was found after RTX therapy.



**Figure 2.** Vλ gene usage and Jλ gene usage in RA. A. Vλ family gene usage was not substantially influenced by rituximab (RTX) therapy. B. Usage of Jλ genes after RTX was not affected by therapy. C. No significant change of usage of distal Vλ genes was found after RTX. D. Comparison of mutational frequencies between Vλ gene rearrangements using Jλ1-3 and those using Jλ7 before (BL, baseline) and after (RTX) therapy.

3-7.01 were expressed at lower frequencies before therapy, but were not found after therapy. By contrast, the genes Vκ1-13.01, 1-13.02, 1-16.01, 2-24.01, 2-30.01, 2-40.01, and 5-2.01 were not detected before depletion, but occurred in the regenerating repertoire (Figure 3A). As expected, most of the CD27<sup>-</sup> naive B cells were unmutated, with a small number of sequences carrying very low mutations (21 sequences with 1–3 mutations, 2 sequences with 6–10 mutations out of 172 sequences).

Usage of individual Vλ genes among naive B cells revealed a predominant expression of Vλ1-44.01 and 2-14.04. All genes observed before therapy were also identified in the reemerging repertoire. In addition, several genes such as Vλ1-51.01, 2-11.03, 3-9.01, 3-21.01, and 9-49.01 that were not found before therapy were identified after therapy. However, the frequency of occurrence of Vλ3-21.01 was significantly higher after RTX compared to baseline ( $p = 0.01$ ). With the exception of Vλ3-21.01, there was no statistically significant difference of Vλ gene usage within naive B cells.

*Usage of Ig Vκ and Vλ minigenes in CD19<sup>+</sup>/CD27<sup>+</sup> mem-*

*ory B cell repertoire.* Analysis of the CD27<sup>+</sup> memory B cell compartment reflecting the superimposed picture of B cell activation, differentiation, and selection provided further interesting insights. In terms of individual Vκ gene usage, Vκ1-5.03, 1-39.01, and 4-1.01 genes were highly expressed before as well as after therapy. Several genes were found at lower or enhanced frequencies at the 2 timepoints of analysis, among which usage of 1-5.03 and 2-30.01 were significant after RTX (Figure 3).

Examination of individual Vλ genes revealed a wide array of genes constituting the repertoire before and after depletion, i.e., Vλ1-40.01, 1-44.01, 1-47.01, 2-8.01, 2-14.01, 2-23.01, 3-21.02, and 6-57.01. Some genes (Vλ1-40.03, 2-23.02, 3-9.01, 3-12.02, 3-25.01, 3-25.03, 5-39.01, and 8-61.01) that were not found in circulation before therapy were detected in the recovery phase, albeit at lower levels, except a few significant differences (Figure 3D) of occurrence of individual Vλ minigenes before and after therapy.

*Imprints of potential receptor revision: usage of Jκ proximal and Jκ distal V gene cassette.* Receptor revision occurs

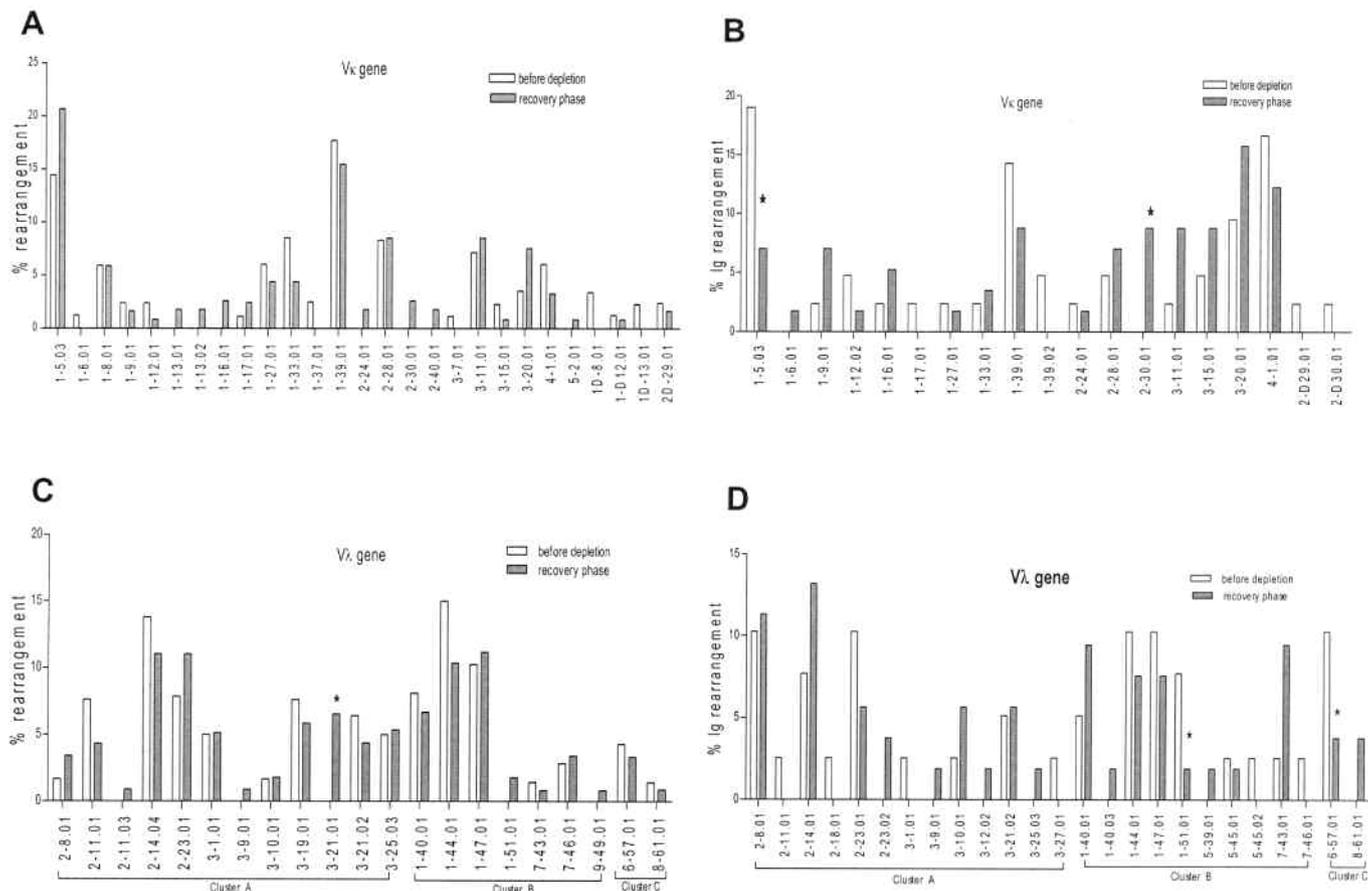


Figure 3. Light chain family minigene distribution in CD19+/CD27<sup>-</sup> (A, C) and CD19+/CD27<sup>+</sup> (B, D) B cells before and after B cell depletion. Panels A and B represent V $\kappa$  family minigene distribution before and after transient B cell depletion in naive (A) and memory B cells (B). Panels C and D display V $\lambda$  gene usage in naive and memory B cells, respectively. \* $p \leq 0.01$  compared to before therapy, 2-tailed Fisher exact test.

mainly at the I $\kappa$ k locus through V $\kappa$  to J $\kappa$  secondary rearrangement. We further investigated whether the use of V genes between the proximal versus distal cassette and their rearrangements to particular J $\kappa$  elements differed, which may reflect active receptor revision. The J $\kappa$  proximal cassette is arranged when most rearrangements occur by deletion, whereas the J $\kappa$  distal cassette is arranged during recombination processes by inversion and in opposite orientation to that of the proximal cassette. Among J $\kappa$  genes, J $\kappa$ 5 gene is most distally located. Analysis of J $\kappa$  gene usage (Figure 1B) revealed that J $\kappa$ 2 was used most frequently before and after RTX. Moreover, RTX led to enhanced J $\kappa$ 1 usage (15% vs 22%;  $p = 0.05$ ) and a reduced frequency of J $\kappa$ 4 gene usage (13% before vs 6% after RTX;  $p < 0.025$ ). Because receptor revision leads to greater usage of V $\kappa$  genes from J $\kappa$  distal cassettes, the frequency of these genes was assessed. Although the overall use of J $\kappa$ 5 gene segments (as an important indicator for active receptor revision) was not substantially different between the 2 timepoints (Figure 1B), RTX treatment led to significantly reduced usage of distal V $\kappa$  genes (2.5% compared to baseline (14%;  $p = 0.0006$ ; Figure 1C). This indicated that the imprints of receptor revision

were likely more active at baseline, compared to the period after RTX. Consistent with this, the mutational frequencies between V $\kappa$  rearrangements of CD19+/CD27+ memory B cells employing J $\kappa$ 5 were significantly lower than those using J $\kappa$ 1-4 ( $p = 0.02$ ; Figure 1D).

*Usage of V $\lambda$  minigenes according to their V $\lambda$  clusters and J $\lambda$  gene usage.* Further studies investigated to what extent the usage of certain V $\lambda$  and J $\lambda$  genes may indicate a different role of receptor revision before and after RTX. Therefore, we analyzed V $\lambda$  gene usage in both subsets according to V $\lambda$  gene clusters. In this regard, V $\lambda$  genes are segregated into 3 clusters, A, B, and C, cluster A being the most J $\lambda$  proximal and cluster C being the most J $\lambda$  distal segment<sup>20</sup>. Notably, the frequencies of V $\lambda$  gene usage residing in cluster A, B, and C genes were comparable at baseline and during the reconstitution phase after RTX (Figure 3C, 3D). Consistent with the V $\kappa$  analysis, the usage of distal V $\lambda$  genes was reduced after RTX therapy (22% at baseline vs 16% after RTX;  $p = 0.147$ ; Figure 2C), indicating that receptor revision was likely more active at baseline.

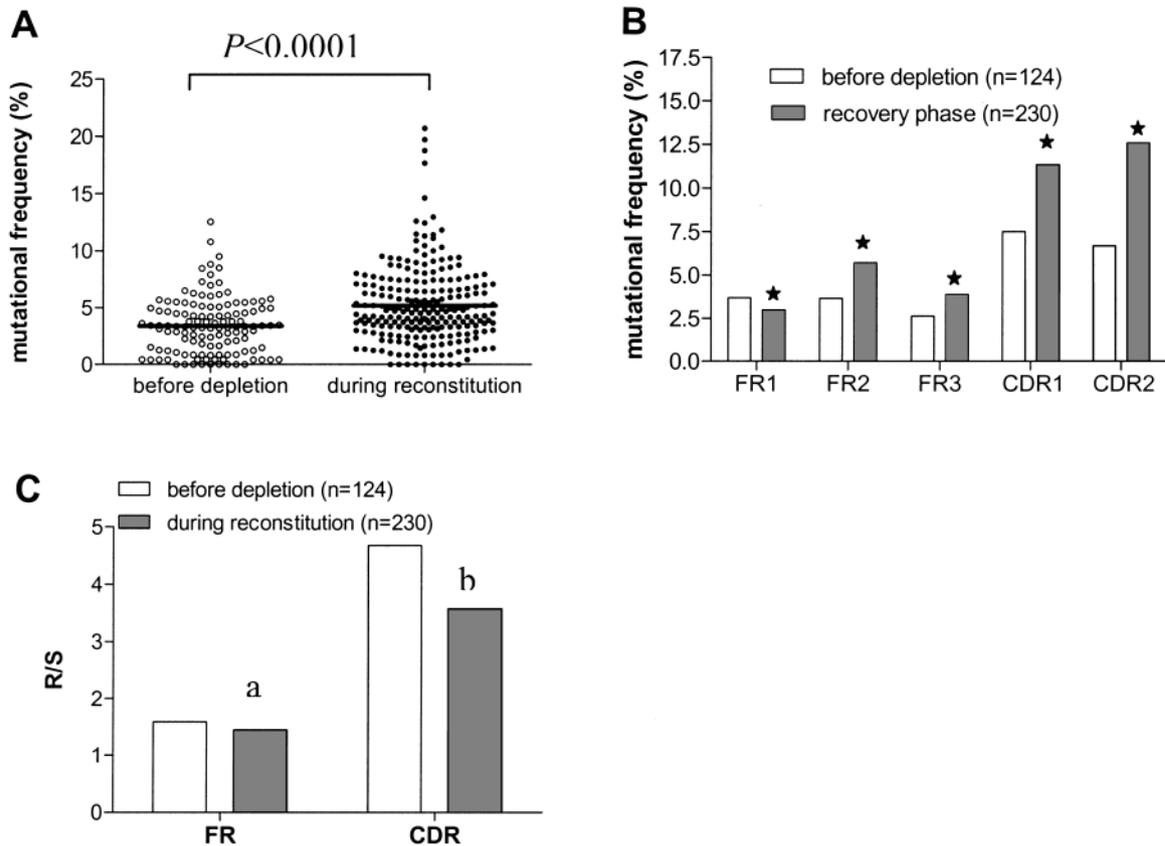
The frequency of usage of J $\lambda$  elements was also comparable at baseline and after RTX, with moderate variations as

indicated by an increase of  $J\lambda 1, 2,$  and  $3$  gene usage and decreased use of  $J\lambda 7$  genes (not statistically significant; Figure 2B). These data indicated that B cell depletion by RTX led to some changes in  $V\lambda/J\lambda$  gene usage; however, these were not significant. Moreover, analysis of the mutational frequency of  $J\lambda 1-3$  containing  $V\lambda$  rearrangements was comparable to those rearrangements employing  $J\lambda 7$  (Figure 2D). Together, the data indicate that there were no striking changes of receptor revision of  $V\lambda$  expressing B cells in these patients with RA undergoing RTX treatment.

**Frequency of somatic hypermutation in  $V\lambda$  and  $V\kappa$  repertoire of CD27+ memory B cells.** There were substantial differences in the mutational frequency of the CD19+/CD27+ memory repertoire before compared to after anti-CD20 therapy. As expected, nearly all of the CD27+ sequences were mutated, with comparable mutational frequencies of the  $V\kappa$  and  $V\lambda$  repertoires. Notably, CD27+ memory B cells showed an almost 2-fold increase of the mutational frequency of  $V\lambda$  and  $V\kappa$  rearrangements after RTX therapy (5.6%; Figure 4A) compared to that before therapy (3.2%;  $p <$

0.0001), with  $V\kappa$  3.2%  $\pm$  0.9% at baseline versus 5.6%  $\pm$  0.1% in the repletion phase and  $V\lambda$  3.1%  $\pm$  0.1% versus 5.6%  $\pm$  0.1%. This increment of the mutational frequency was related to enhanced mutations residing in framework regions (FR 1 to 3) as well as complementary determining regions (CDR 1 and 2; Figure 4B, 4C). Further examination of the expression of mutations in the CDR and framework regions showed a reduced ratio of replacement to silent mutations (R/S) in the CDR ( $p = 0.001$ ) and in the FR ( $p = 0.04$ ) after treatment. No differences were observed in the frequency of transitions versus transversions or in the RGYW/WRCY hotspot motif (data not shown) targeted during B cell repletion.

**CDR3 length.** The overall CDR3 lengths were 30.0  $\pm$  0.27 and 27.0  $\pm$  0.16 (median  $\pm$  SEM) within the  $V\kappa$  rearrangements by naive and memory B cell rearrangements at baseline, respectively. These CDR3 lengths remained unaltered after anti-B cell therapy (30.0  $\pm$  0.31 for naive B cells and 27.0  $\pm$  0.26 for memory B cells). As well, the CDR3 lengths of  $V\lambda$  rearrangements were similar in both naive and mem-



**Figure 4.** Pattern of mutations in the CD19+/CD27+ repertoire. A. Ig mutational frequencies of single CD19+/CD27+ B cells from the  $V_L$  repertoire before B cell depletion and during recovery. Recovering B cells exhibit an increased mutational frequency compared to status before depletion ( $p = 0.0001$ , chi-square test). B. Analysis of mutational frequencies in framework regions (FR) 1-3 and complementary determining regions (CDR) 1 and 2 displayed elevated mutational frequencies in CDR1 and 2 before depletion and during recovery. B cell depletion induced changes in mutational frequencies in the FR and CDR. Regenerating B cells displayed decreased FR1 mutational frequency but increased FR2, FR3, CDR1, and CDR2 frequencies. All changes were statistically significant ( $p = 0.0001$ , chi-square test). C. R/S mutations are diminished in the FR and CDR of CD27+ VL repertoire (a:  $p = 0.04$ ; b:  $p = 0.001$ , chi-square test).

ory B cell subsets ( $33.0 \pm 0.34$  vs  $30.0 \pm 0.26$  in naive;  $27.0 \pm 0.40$  vs  $30.0 \pm 0.30$  in memory B cells) before and after RTX. The lack of changes in CDR3 lengths suggests that selective pressures effective on the CDR3 length were less strikingly different before and after RTX therapy.

## DISCUSSION

B cell depletion by the anti-CD20 antibody RTX has proven to be an efficacious and comparably safe treatment in patients with moderate to severe RA as well as other autoimmune diseases<sup>7,8,21</sup>, supporting the role of B cells in their pathophysiology. RTX-mediated B cell depletion yields a longterm effect on immunological homeostasis including distinct peripheral blood B cell subpopulations with high numbers of transitional B cells<sup>8</sup>, indicating recapitulation of B cell ontogeny. Although numeric normalization of B cell numbers takes place in the majority of patients between 1 and 2 years after a single course of RTX, a longterm delay in memory B cell subsets has been observed especially in responders<sup>8,9</sup>. Gene rearrangement analysis of IgV using single B cells allows study of different processes such as somatic hypermutation and receptor revision. Abnormalities in these events alter the composition of the peripheral B cell repertoire. Previous observations provided evidence of the influence of RTX on molecular imprints shaping the Ig heavy chain receptor<sup>15,22,23</sup>, but did not allow any evaluation of receptor revision. Receptor revision together with the imprints of somatic hypermutation, CDR3 length, and Ig switching represent results of T cell and antigen-dependent B cell activation and differentiation, which also reflect molecular signatures of immune memory. To date, modulatory effects in the immunoglobulin light chain repertoire after RTX have not been studied in detail. Therefore in this study we investigated the effects of RTX on the V $\kappa$  and V $\lambda$  light chain repertoire genes in the CD27<sup>-</sup> and CD27<sup>+</sup> B cell compartment in patients with active RA, using single B cell analyses before and during B cell repopulation after RTX.

Different mechanisms shaping the human light chain repertoire including selection and combinational biases serve the limitation of expressed repertoire. Repertoire revision in B cells and the clonal selection processes contribute to somatic evolution of appropriate immune responses under normal circumstances<sup>6</sup>. Examination of individual V $\kappa$  minigene distribution revealed a diverse repertoire of genes, with specific V $\kappa$ 1 family members being predominantly expanded in repopulating B cells. A similar overrepresentation of V $\kappa$ 1 family members has also been reported in cDNA libraries studied from synovial tissue of a patient with RA in comparison to healthy donors<sup>24</sup>. A restricted repertoire of V $\kappa$  has been reported in patients with juvenile arthritis<sup>11</sup>. Interestingly, we did not find a restricted repertoire of V $\kappa$  family gene usage in our patients with RA, which may indicate an age-dependent modulation (Figure 1A, 1B). The V $\kappa$  family repertoire changed significantly after RTX (Figure

3A) as indicated by a decrease in the use of V $\kappa$ 1 and an increase in V $\kappa$ 3 families. Interesting as well, the B3 gene, a member of the V $\kappa$ 4 family, previously shown to be overexpressed in patients with systemic lupus erythematosus (SLE)<sup>25</sup> and linked to encode autoantibodies<sup>26</sup>, was not used by our 2 patients with RA, consistent with a differential usage of V $\kappa$  minigenes during flares of different autoimmune diseases. Analysis of the V $\lambda$  repertoire showed the majority of minigenes in equal distributions after RTX, except V $\lambda$ 3-21.01, which was used significantly more ( $p = 0.01$ ) in repopulated CD27<sup>-</sup> B cells, while 1-51.01 and 6-57.01 were reduced in CD27<sup>+</sup> B cells after RTX. These findings indicate that a diverse and polyclonal V $\kappa$  and V $\lambda$  gene repertoire is emerging after RTX-induced temporal B cell depletion, suggesting a reestablishment of B cell homeostasis.

There is controversy whether defective mechanisms in secondary gene rearrangements are involved in generating autoimmunity. Studies indicate that receptor revision is a principal mechanism of B cell tolerance<sup>12</sup> and defective central or peripheral receptor revision has been linked to autoimmunity<sup>27</sup>. The frequency of J-distal V $\kappa$  gene usage was significantly less than J-proximal V $\kappa$  genes after therapy (Figure 1C). These data indicate that imprints of receptor revision are already evident before RTX, and therapy-related changes do not support a potential defective receptor revision in patients with RA. Two different molecular mechanisms are operative on V $\kappa$ /J $\kappa$  rearrangements, one by inversion and another by deletion. Inversion is believed to be the significantly less efficient process, suggested by artificial recombination substrate studies<sup>28,29</sup>. Recombination requires rearrangement over a long stretch of DNA because the J-distal cassette is 1500 to 2000 kb from the nearest J $\kappa$  gene, with possibly less efficient recombination machinery over distances  $> 1000$  kb. While no firm conclusion can be drawn about the exact mechanisms applied by patients with RA, the current data for significantly reduced usage of J $\kappa$ -distal genes in repopulated B cells suggest that RTX therapy normalizes B cell homeostasis by reducing the imprints of receptor revision, which may have been active particularly on V $\kappa$ J $\kappa$  gene rearrangements. Because of the distal location of J $\kappa$ 5, a diminished mutational frequency was observed in memory B cell rearrangements compared to those using J $\kappa$ 1-4 (Figure 1D) at baseline. Together, the data indicate that receptor revision took place at baseline on the V $\kappa$  locus, excluding the possibility of a defective receptor revision process in these patients with RA. Moreover, receptor revision in V $\kappa$  probably occurred in secondary lymphoid organs after initiation of somatic hypermutation, as suggested by fewer mutations in rearrangements using J $\kappa$ 5.

Analysis of the V $\lambda$  gene repertoire did not reveal significant differences after RTX. The overall usage of J-distal V $\lambda$  genes was decreased without reaching statistical significance (Figure 4C), indicating that the effects of RTX on V $\lambda$

gene rearrangements were less striking compared to the V $\kappa$  locus. This is consistent with the conclusion that receptor revision in V $\lambda$  occurred centrally at baseline.

We did not find increased usage of cluster C genes, in contrast to previous findings in a patient with SLE<sup>30</sup>. Increased use of cluster C genes is believed to be a predictor of extensive receptor revision in V $\lambda$  gene rearrangements because it is also observed in infants compared to healthy subjects<sup>31</sup>. Cluster C genes are most J $\lambda$  distally located with more than 600 kb upstream of J $\lambda$ 1 locus. In our patients with RA, cluster A and B genes were frequently used, consistent with findings from healthy donors<sup>31</sup>. Only moderate changes were observed in the usage of J $\lambda$  genes during therapy (Figure 2B), suggesting that the basic molecular mechanisms driving recombination and selection of V $\lambda$  chain rearrangements were not substantially altered by RTX.

We noted profound changes in the CD27+ memory B cells during B cell repopulation after RTX. The mutational pattern of repopulated CD27+ memory B cells was marked by the prevalence of highly mutated V $\kappa$  and V $\lambda$  Ig gene sequences (Figure 4A). These findings are in accord with our previous findings, where we analyzed the mutational pattern of Ig-V<sub>H</sub>4 gene in a cohort of RA patients treated with RTX<sup>15</sup>, and support the concept that RTX-mediated B cell depletion modulates Ig heavy and light chains. It is assumed that repopulation of the peripheral repertoire is mainly derived from early undepleted CD20-negative pro-B cells, and permits early occurrence of highly mutated sequences during B cell repopulation. These highly mutated cells during the early regeneration phase might develop from a newly generated B cell pool that rapidly undergoes intensive somatic hypermutation and circulates in the periphery. However, part of the highly mutated memory pool may also be dependent on surviving cells, which can be found in the periphery in low frequency during the depletion phase and may generate from protected niches<sup>32,33</sup>. The increased mutational frequency by regenerated memory B cells can be explained by a marked reduction in pre-switch memory B cells and a relative increase of recirculating plasmablasts during the regeneration phase (Table 1). Detailed analyses of CDR (Figure 4B) showed that the V $\lambda$  and V $\kappa$  repertoire exhibited increased mutational frequency in the CDR2, followed by CDR1, before and after depletion. Similar observations were noted within framework regions. This indicates that B cell depletion by RTX introduced significant modulations in the regenerating V $\lambda$ /V $\kappa$  repertoire. Of note, the ratio of replacement to silent mutations (R/S) in the complementary determining regions and framework regions revealed that RTX induced modulations of R/S ratios. We observed a significantly decreased R/S mutational ratio after RTX (R/S CDR and FR in V<sub>L</sub>:  $p = 0.001$ ,  $p = 0.04$ , baseline vs reconstitution phase, respectively). Positive selection of R mutations has been reported for V<sub>H</sub> and V<sub>L</sub> gene rearrangements in healthy individuals<sup>34,35,36</sup>,

and therefore the reduction in R/S mutations may also be indicative of lower affinity of potential autoreactive clones that recirculate during B cell reconstitution in patients with RA.

Overall, this is to our knowledge the first study to report the regeneration pattern of Ig V $\kappa$  and V $\lambda$  light chain gene rearrangements during RTX therapy. Receptor revision was operative in these patients mainly on the V $\kappa$  locus at baseline. Transient B cell depletion introduced differential V $\kappa$ J $\kappa$  and V $\lambda$ J $\lambda$  minigene usage in the regenerating naive and memory B cell repertoires. Memory B cells were substantially marked by an elevated mutational frequency of the reemerging CD27+ light chain. While our findings do not support defects of receptor revision, at least on the V $\kappa$  locus in these patients with RA, the data provide evidence that anti-CD20 therapy probably alters the molecular characteristics of the V light chain gene repertoire consistent with a reduction of receptor revision.

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