Analysis of RAG expression by peripheral blood CD5+ and CD5- B cells of patients with childhood Systemic Lupus Erythematosus

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Abstract

Background: The assembly of immunoglobulin genes during B cell development in the bone marrow is dependent on the expression of recombination activating genes (RAG) 1 and 2. Recently, RAG expression in peripheral blood IgD+ B cells outside the bone marrow has been demonstrated and associated with the development of autoimmune diseases.

Objective, Methods: By using a combination of flow cytometric cell sorting and RT-PCR analysis of cDNA libraries generated from individual cells, the expression of RAG, VpreB and CD154 mRNA by individual peripheral blood B cells of three paediatric SLE patients was examined in detail.

Results: Whereas only one patient indicated a significantly higher frequency of RAG+ B cells in the CD5- B cell population all patients showed significantly higher frequencies of RAG+ B cells in the CD5+IgD+ B cell population. The frequency of RAG+ IgD+CD5+-/- B cells was reduced during i.v. cyclophosphamide therapy. In healthy, age-matched individuals RAG expressing IgD+ B cells were hardly detectable. Coexpression of RAG and VpreB or CD154 mRNA could only be found in SLE B cells.

Conclusion: RAG expression in peripheral blood B cells of SLE patients is particularly increased in the IgD+CD5+ B cell population. CD5+ and CD5- B cells in SLE have the potential to undergo receptor revision leading to the generation of high-affinity, pathogenic autoantibodies.
Key words
Recombination activating genes
CD5+ B cells
Systemic lupus erythematosus
Receptor Editing
Receptor Revision
Introduction
Systemic lupus erythematosus (SLE) is an autoimmune disease, affecting both adults and children. Although childhood SLE resembles adult SLE concerning presentation, clinical findings and pathogenesis, children seem to have a more severe disease at onset with higher rates of organ involvement as well as a more aggressive clinical course (1). SLE is characterized by a wide range of abnormalities of the immune system and multi-organ tissue pathology (2, 3). High affinity autoantibodies to dsDNA which are produced by autoreactive B cells are one of the diagnostic criteria of SLE (4). They play a central role in the induction of tissue damage, especially of lupus glomerulonephritis. The molecular process leading to the generation of autoreactive B cell receptors (BCR) however is still unknown.

B cells assemble the coding region of their immunoglobulin receptor during their development in the bone marrow (5). The process of V(D)J recombination is dependent on the coordinated expression of RAG proteins 1 and 2, which are encoded by the recombination-activating genes 1 and 2 (RAG) (6, 7). These enzymes mediate the initial DNA breaks in variable (V), diversity (D) and joining (J) gene segments (8). Recent data show that a significant number of early immature B cells bear an autoreactive receptor after the first V(D)J recombination (9). Besides apoptotic deletion and the generation of B cell anergy (10), revision of this autoreactive receptor by another cycle of V(D)J recombination in the bone marrow, called receptor editing, is considered to be a mechanism to prevent autoimmunity (11-14). It has been shown that receptor editing in the bone marrow prohibits autoimmunity in transgenic animals and it appears to be a powerful mechanism to protect humans from autoimmunity (9, 11, 13-20). Until recently, RAG expression and V(D)J recombination were thought to appear solely in immature, developing B cells in the bone marrow. However, we and others have detected RAG 1 and 2 expression in germinal center B cells in secondary lymphoid organs of mice and humans (21-27). Only small populations of normal human B cells in the peripheral blood have been reported to express RAG mRNA. Recently, we could demonstrate an increase of coordinate RAG 1 and 2 mRNA expression in peripheral blood B cells of SLE patients (22). Receptor editing in the bone marrow and receptor revision in the periphery seem to have different biological functions. Whereas the former mechanism seems to be tolerance driven, the latter rather seems to diversify the immunoglobulin repertoire, thereby potentially generating autoreactive B cell receptors (1, 28, 29).

VpreB is an essential part of the surrogate light chain. Expression is normally restricted to B cell development in the bone marrow during early light chain rearrangement (30). However, an increased expression of surface VpreB and VpreB mRNA could be detected in peripheral blood B cells of patients with SLE and other autoimmune diseases and might be an indicator of ongoing or reactivated V(D)J recombination (22, 31, 32).

CD154, the ligand of the CD40 receptor, is normally expressed on activated T cells during germinal center reactions thereby giving help to activated B cells (33, 34). In contrast, CD154 (CD40L) mRNA expression in peripheral blood SLE B cells demonstrates activation of these B cells. RAG expression in peripheral SLE B cells has been associated with CD154 mRNA expression (22, 35).

B cells can be subdivided into two subpopulations regarding their expression of CD5: B-1 B cells, which are mainly CD5+ and conventional B-2 B cells lacking surface expression of CD5 (36). B-1 B cells are known to produce low affinity polyreactive antibodies, which recognize autoantigens or conserved structures on self-antigens, such as polysaccharide residues (37). There is evidence that CD5+ B cells might
play a role in the pathogenesis of autoimmune disease (38). However, the pathogenic impact of CD5+ B cells in SLE still remains unclear. Recently, increased expression of RAG in peritoneal B cells of NZB mice, a murine model for human SLE, was detected (39). This suggested that receptor revision outside the bone marrow could be an ongoing molecular process in B-1 B cells, which potentially could contribute to the formation of an autoreactive B-1 B cell repertoire.

In this study we investigated RAG expression in the CD5+ or CD5- IgD+ B cell compartment in childhood SLE patients.
Patients and Methods

Preparation of B cells from peripheral blood
Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-Hypaque density gradient centrifugation from heparinised peripheral blood of 3 subjects with active SLE and 2 healthy donors. The subjects fulfilled the revised ACR criteria for classification of SLE (4). At the time of analysis, subject 1 (male, 11 years) presented with type IV glomerulonephritis and thrombocytopenia, subject 2 (female, 18 years) with thrombocytopenia and subject 3 (female, 13 years) with type II glomerulonephritis and a malar rash. All patients were ANA and anti-double-stranded DNA antibody positive. Anti ds-DNA antibodies were measured using a radioimmunoassay (RIA), an ELISA and an immunofluorescence test. ANA antibody (ANA) titres were analyzed using an immunofluorescence test. PBMCs of subject 1 were separated before, after one, two and seven i.v. cyclophosphamide treatments. A total of ten treatments were given. In this patient, absence of clinical symptoms and normalization of laboratory values was achieved after 1 year (7th cyclophosphamide pulse). However, the anti-DNA and ANA titers did not change during and after discontinuation of i.v. cyclophosphamide. In the other subjects, we obtained two blood samples each during the disease course and calculated the mean of the two. Both subjects showed only minor haematological abnormalities at these follow-up time points, while being treated with low dose glucocorticoids and hydroxychloroquine. Both healthy individuals (10 year-old female, 16 year-old male) were healthy and did not show any sign of autoimmune feature or infection. The study was approved by the ethics committee of the University of Würzburg. Written consent was obtained from each patient or parents.

Single-cell sorting and Flow Cytometric Analysis
To obtain single B cells, PBMCs were stained for the surface expression of CD19 in combination with IgD and CD5 for 30 minutes using anti-human CD19 (Tri color-labeled, Caltag, Burlingame, CA, USA), anti-human IgD (fluorescein isothiocyanate-labeled, Caltag) and CD5 (phycoerythrin-labeled, Caltag) antibodies. Three-colour immunofluorescence analysis was used for identification of the different B cell populations. Isotype-matched antibodies were used as controls. FACS analysis was performed with a FACStar flow cytometer using CellQuest software (Becton Dickinson, Franklin Lakes, NJ, USA).
Individual CD19+IgD+CD5+ and CD19+IgD+CD5- B cells of the SLE patients were sorted into 96-well polymerase chain reaction (PCR) plates using a FACS Vantage flow cytometer (Becton Dickinson) outfitted with a single-cell deposition unit, as described before (21, 22).

Preparation of RNA and cDNA from sorted single cells
Ten microliters of lysis solution (2µl of 5X first strand buffer (invitrogen, Karlsruhe, Germany), 10mM dithiotreitol (invitrogen), 1% Nonidet-NP40 (Sigma, St. Louis, MO), 10 units of recombinant Rnasin ribonuclease inhibitor (Promega, Madison, WI), 0.8mM of each dATP, dCTP, dGTP, dTTP (Sigma), 0.1 µg oligo d(T)12-18 (Amersham Pharmacia Biotec, Piscataway, NJ) were added into each well of the PCR plate before sorting individual cells into the wells. The conversion of mRNA to cDNA of these individual cells was carried out using Superscript II Rnase H – reverse transcriptase (invitrogen) as described previously. By this method a cDNA library was generated from individual cells allowing the analysis of coexpression of up to 10 different genes of interest.
PCR amplification
PCR amplification of cDNA generated from single cells specific for β-actin, RAG1, RAG2, VpreB and CD154 was carried out in two rounds, using external and nested primers as listed in table 1. PCR amplification conditions were as described previously. In short, for the analysis of human RAG2 cDNA, two different 5’ primers for the alternative exon 2A and exon 2B were used. Control samples without adding cDNA or RT were run in parallel and did not yield a product. RAG amplification from genomic DNA was excluded as described in previous experiments (21, 22).

Detection of amplified cDNA by Southern Blot analysis
PCR products were transferred to a nylon membrane by the alkaline Dot Blot procedure (Biorad). PCR Dot Blots were incubated in hybridization buffer containing DIG-dUTP-labeled probes (Roche, Mannheim, Germany) specific for the targeted PCR products. DIG-dUTP was detected in a chemiluminescent reaction, using alkaline phosphatase coupled anti-DIG-dUTP antibody and CSPD. Visualization was obtained by exposure to a photographic film. The blots were analyzed on a digital detection unit (Biorad, Hercules, CA, USA) using QuantityOne Software (Biorad). Only beta-actin positive cells were considered for the analysis of RAG1 and2, VpreB or CD154 mRNA expression. The number of the cells analyzed in each patient are shown in table 2.

Statistical analysis
Statistical analysis for comparison of single cell gene expression patterns of different B cell populations was performed using χ² test.
Results

Both CD5+ and CD5- IgD+ peripheral blood B cells show coordinated expression of RAG1 and RAG2 mRNA in SLE, which is particularly upregulated in CD5+ B cells when compared to healthy controls

Since the functionality of the V(D)J-recombinase RAG is dependent on the assembly of both subunits, RAG1 and RAG2, we calculated the frequency of cells expressing RAG1 as well as RAG2 mRNA (RAG+ cells) in a defined population. Since IgD+ B cells seemed to be the population with highest frequencies of RAG+ cells in SLE (22), we were interested to characterize this population more in detail.

As expected from previous reports only low frequencies of RAG+ B cells could be found in the peripheral blood of healthy individuals. Notably, CD5- B cells expressed RAG 1/2 mRNA at a frequency of 2.5 % (Figure 1), whereas no coordinate expression of RAG1/2 mRNA could be found in the CD5+ population of two healthy donors. RAG 1/2 mRNA expression was upregulated in peripheral IgD+ B cells of SLE patients (range 0.9 – 30%). We could not find significant differences in the frequency of RAG+ cells between CD5+ and CD5- B cells within each of the three patients analysed. However, when we compared the frequency of RAG+ cells in each CD5+ or - population of SLE patients with their counterpart in healthy age matched individuals, significant differences were obvious (Figure 1a). All three SLE patients showed significantly higher frequencies of RAG+ cells in the CD5+ B cell population as compared to the CD5+ B cells in healthy individuals. However, in the CD5- B cell population only one patient showed increased frequencies of RAG+ cells when compared to healthy individuals (Figure 1a).

Interestingly, interindividual differences in the frequency of RAG+ B cells could be found between the analysed patients. The highest frequency of RAG+ B cells could be found in the patient with highest disease activity (type IV glomerulonephritis, thrombocytopenia, SLEDAI total score of 21; Patient 1) (Figure 1b).

The frequency of CD154 or VpreB mRNA expressing cells was increased in both CD5+ and CD5- IgD+ SLE B cells

To analyse, if peripheral blood CD5+ and CD5- B cells of SLE patients differed in their expression of VpreB or CD154 mRNA, we detected mRNA expression of these markers on a single cell level in both populations of patient 1, who showed highest disease activity.

Healthy individuals showed a moderate frequency of VpreB mRNA expressing cells in both the CD5+ and CD5- B cell population (5.8%, 4.2% resp.) (Figure 2a). The frequency of VpreB+ B cells was significantly increased in both CD5+ and CD5- B cells of this SLE patient as compared to healthy individuals (18%, p<0.05; 30%, p<0.01 resp.). However, neither significant differences in the frequency of VpreB+ cells between the CD5+ and CD5- population within the SLE patient (p>0.05) nor a characteristically dominant increase of VpreB mRNA expression in one particular of these populations as compared to healthy individuals could be noted.

Similar patterns were analysed for the expression of CD154 mRNA (Figure 2a). The frequency of CD154 mRNA expressing cells was significantly higher in both CD5+ and CD5- B cells of this SLE patient (24%, p<0.01; 20%, p<0.05 resp.) compared to healthy individuals (3.3%, 4.2% resp.). No differences regarding the expression of CD154 mRNA could be found between the two analysed cell populations (p>0.05).
Peripheral blood IgD+CD5+/− SLE B cells show increased coexpression of RAG1/2 and CD154 or VpreB mRNA

Peripheral blood IgD+ B cells coexpressing RAG1/2 and VpreB mRNA could readily be found in SLE patient 1 in the CD5+ (4%) as well as in the CD5− B cell population (10%). However, statistical analysis did not reach significance when expression frequencies were compared in between CD5+ and CD5− B cells (p>0.05). Of note, they were not detectable in healthy individuals (0%, 0%) (Figure 2b).

Whereas 2% of all CD5+ and 10% of all CD5− peripheral IgD+ B cells in SLE patient 1 coexpressed RAG1/2 and CD154 mRNA, no coexpression of these markers could be found in these cell populations of healthy individuals. The differences between CD5+ and CD5− B cells in the patient reached no significance (p>0.05) (Figure 2b).

CD5+ RAG+ B cells were most sensitive to i.v. cyclophosphamide treatment

To test the hypothesis that the presence of RAG+CD5+ as well as RAG+VpreB+ or RAG+CD154+ B cells in the peripheral blood of SLE patients are relevant to disease activity, we analysed whether these cells react differently during i.v. cyclophosphamide treatment due to glomerulonephritis type IV.

Whereas the frequency of RAG+ B cells in the CD5+ population decreased significantly after the first treatment (p<0.01), the frequency of RAG+ cells in the CD5− population stayed stable or even increased and did not reach significantly lower levels (p<0.01) before the 7th i.v. cyclophosphamide treatment (Figure 3a). Notably, also the frequency of CD154+ or VpreB+ cells in the RAG+ B cell population was reduced after one i.v. cyclophosphamide treatment in both CD5+ and CD5− CD19+ IgD+ B cells in contrast to those cells expressing only CD154 or VpreB mRNA (data not shown). Proteinuria as well as SLEDAI score decreased in this patient after the first i.v. cyclophosphamide treatment and absence of clinical symptoms was achieved after the 7th treatment (Figure 2a). Interestingly, after the 7th cyclophosphamide treatment the IgD+ B cell population was dominated mainly by CD5− B cells which did not express RAG1/2 and CD154 or VpreB (Figure 2b).
Discussion

RAG expression is a crucial and tightly regulated event during B cell development in the bone marrow enabling the assembly of immunoglobulin genes in an almost random process of V(D)J recombination (5, 6). B cell development seems to be under control of several central B cell tolerance mechanisms thereby avoiding the generation and/or emigration of autoreactive B cells. V(D)J recombination is restricted to B cell development in the bone marrow and RAG expression is almost absent in peripheral blood B cells of healthy individuals. Increased RAG expression outside the bone marrow might lead to uncontrolled V(D)J-recombination and eventually to a shift of the BCR repertoire towards autoreactivity.

In this study we analyzed the frequency of RAG expressing cells in the CD19+IgD+ CD5+ and CD5- peripheral blood B cell population of three juvenile SLE patients and two age matched healthy individuals. In healthy individuals, RAG expression was almost absent in peripheral blood B cells and could only be found in low levels in the CD5- B cell population. This is consistent with data of a previous study showing peripheral RAG expression in adolescent healthy mice only in the CD5- but not in the CD5+ B cell population (40). Interestingly, whereas only one patient in this study indicated significantly higher frequencies of RAG+ B cells in the CD5+ B cell population all patients showed significantly higher frequencies of RAG+ B cells in the CD5+ population. Therefore, one might conclude that RAG expression seems to be upregulated in particular in the CD5+ B cell population in SLE patients. This is supported by experiments analyzing RAG expression in murine lupus (39).

Recently, we showed increased expression of RAG mRNA in peripheral B cells of adult SLE patients. This observation as well as the data in juvenile SLE patients from this study might support the assumption of a similar pathogenesis of childhood and adult SLE.

It is not clear which mechanisms contribute to the increased frequency of RAG expressing B cells in the peripheral blood of SLE patients. These B cells might either re-express RAG at mature stages of B cell differentiation or bone marrow emigration of immature B cells still expressing RAG might be increased.

Re-expression of RAG in peripheral mature B cells in vitro or during germinal centre reactions was initially observed after activation of these cells by antigen challenge or BCR crosslinking (23-25). CD5+ B1 B cells normally bear low affinity auto-/polyreactive BCRs (36). Since clearance of apoptotic cells is impaired in SLE patients, huge amounts of autoantigens might be presented in this context (41). Poly-/auto reactive CD5+ B cells might recognize these autoantigens with low affinity and re-express RAG during cognate activation.

In contrast, the concept of RAG re-expression in peripheral mature B cells was challenged by studies showing that RAG+ peripheral B cells are rather B cell precursors recently emigrated from the bone marrow still expressing some amount of RAG (42). An increased influx of lymphocyte precursors into the peripheral blood seems to be a characteristic feature during inflammation and might therefore explain the appearance of RAG expressing B cells in the peripheral blood in SLE patients.

Supporting this hypothesis we could find increased expression of VpreB mRNA in SLE B cells of the peripheral blood. Surrogate light chain component VpreB is normally expressed during B cell development in the bone marrow and only a small fraction of normal adult peripheral blood B cells have been shown to be VpreB+ (21, 32). On the other hand, expression of surrogate light chain components could be an indicator of ongoing V(D)J recombination in RAG+ B cells, irrespective of their maturational stage (21, 22, 32).
However, observing RAG expression in IgD+ but not in IgG+ SLE B cells (22) as well as the fact that all RAG+ B cells in this study do not coexpress CD27 mRNA (data not shown) assigns these RAG expressing B cells to a rather immature/transitional stage than to the memory B cell compartment. Interestingly, some of these RAG expressing cells in our study showed signs of activation by the co-expression of CD154mRNA (Figure 2). Co-expression of CD154 mRNA and RAG mRNA could not be found in peripheral blood B cells of healthy individuals. However, the phenomenon of coexpression of RAG and CD154 or VpreB mRNA needs further detailed analysis. Independent of the mechanisms which lead to the increased frequency of RAG expressing B cells in the peripheral blood of SLE patients we suggest that increased frequencies of RAG+ IgD+ CD5+ B cells outside the central tolerance mechanisms of the bone marrow seems to be abnormal and might be a pathogenic feature in SLE.

Low affinity, polyreactive antibodies (natural antibodies) are mainly produced by CD5+ B1 B cells (36). It was therefore suggested that immunoglobulin genes of CD5+ B cells might serve as a template for the generation of high-affinity autoantibodies in patients with SLE (43). In this study we could demonstrated increased frequencies of RAG expressing B cells particularly in the IgD+CD5+ B cell population which might be an evidence for ongoing V(D)J recombination in these cells (22, 44). Uncontrolled V(D)J recombination outside the bone marrow might account for a peripheral generation of high-affinity autoreactive B cells in SLE.

In the current analysis we could show that both IgD+ CD5+ and CD5- B cell populations were markedly reduced after two rounds of i.v. cyclophosphamide (Figure 3). Additionally, the frequency of RAG+ B cells in the CD5- population correlated well with disease activity (SLEDAI, proteinuria). Notably, the composition of the IgD+ B cell subset was changed after the 7th cyclophosphamide treatment when absence of clinical symptoms was achieved. The dominating CD5- B cell subset did not show RAG expression. This finding argues for a pathogenic significance of RAG expressing CD5+ peripheral blood B cells in active SLE. However, an increased coordinated RAG expression not only appeared in CD5+ B cells, but also in the CD5- population of patient 1. Additionally, despite of diminished disease activity and reduced RAG+ B cells in the CD5+ population, the frequencies of RAG+ B cells in the CD5- subset even increased during the first i.v. cyclophosphamide treatments. This raises the question whether RAG expression in peripheral blood B cells of patients with high disease activity (e.g. patient 1) might be increased irrespective of CD5 expression.

Although RAG expression and subsequent secondary V(D)J recombination might eventually be involved in the generation of high-affinity autoantibodies in CD5+ B cells, it could be shown, that both CD5+ and CD5- B cells are able to produce anti-dsDNA antibodies (45, 46).

In conclusion, using multivalent immunophenotyping and molecular analysis of cDNA libraries generated from individual cells we have found that increased RAG expression in peripheral blood B cells of SLE patients can mainly be attributed to the IgD+CD5+ B cell population. Low-affinity autoreactive CD5+ B cells might serve as a template for the generation of high affinity, pathogenic autoantibodies by secondary V(D)J recombination.
List of abbreviations
SLE systemic lupus erythematosus
RAG recombination activating genes
PBMCs peripheral blood mononuclear cells
PCR polymerase chain reaction
Ig immunoglobulin
BCR B cell receptor

Competing Interests
None declared

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Literature


Table 1: Sequences of oligonucleotides used as primers for the amplification of cDNA or for the detection of PCR products by Dot blotting

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Figure 1: RAG1 and RAG2A/B mRNA expression in CD5+ and CD5- IgD+ peripheral blood B cells of patients with SLE at the time of diagnosis
Whereas only one patient indicated a significantly higher frequency of RAG+ B cells in the CD5- B cell population all patients showed significantly higher frequencies of RAG+ B cells in the CD5+IgD+ B cell population. The patient with the highest disease activity (SLEDAI) also showed the highest frequency of RAG+ B cells. The frequencies of cells with coordinate expression of RAG1 and RAG2A/B mRNA in individual peripheral CD19+ IgD+ CD5+ (black) and CD5- (white) B cells of 3 SLE patients and healthy individuals (mean of two) are shown. Frequencies (a) of RAG+ cells were compared to the corresponding B cell population in healthy individuals. Absolute cell counts of RAG+ cells are shown in (b). Only beta-actin mRNA positive cells were considered for the analysis of the frequency of RAG1 and RAG2 mRNA expressing cells. Frequencies were calculated as (RAG+ + RAG2+/beta-actin)x100. For didactic means RAG1 and RAG2A/B positive B cells are indicated as RAG+. The disease activity of the three SLE patients is shown as total SLEDAI score (c).

Figure 2: VpreB, CD154 (CD40Ligand) and RAG1/2 mRNA expression in peripheral blood SLE B cells at time of diagnosis
The frequency of CD154 or VpreB mRNA expressing cells was increased in both CD5+ and CD5- IgD+ SLE B cells. Whereas peripheral blood IgD+CD5+/ CD5- SLE B cells show increased coexpression of RAG1/2 and CD154 or VpreB mRNA, no coexpression of these markers could be found in peripheral blood B cells of healthy individuals.
Expression of VpreB, CD154 (CD40Ligand) and RAG mRNA (RAG1 + RAG2) in individual peripheral CD19+IgD+CD5+ (black) and CD5- (white) B cells of healthy individuals (mean of two) and patient 1 was achieved.
a The frequency of cells indicating VpreB mRNA expression was calculated as (VpreB+/beta-actin)x100. The frequency of cells indicating CD154 mRNA expression was calculated in the same way.
b The frequencies of VpreB+ and RAG+ B cells was determined in the two CD5+ and CD5- subsets as (VpreB+ RAG+)/ beta-actin)x100. The frequency of CD154+ and RAG+ B cells was calculated in the same way.

Figure 3: RAG1/2 mRNA expression in peripheral blood SLE B cells at time of diagnosis and during i.v. cyclophosphamide treatment
The frequency of CD5+RAG+ B cells decreased during i.v. cyclophosphamide treatment and correlated with disease activity (SLEDAI), whereas the frequency of RAG+ cells in the CD5- population stayed stable or even increased during the first treatment phase. At the time of absence of clinical symptoms the peripheral blood B cell pool was dominated by CD5- RAG- B cells.
Expression of RAG mRNA (RAG1 + RAG2) in individual peripheral CD19+IgD+CD5+ (black) and CD5- (white) B cells of healthy individuals (mean of two) and patient 1 before, after one, two and 7 cyclophosphamide pulses.
a Frequencies were calculated as (RAG1+RAG2A/B+)/(beta-actin)x100. The disease activity of patient 1 during i.v. cyclophosphamide treatment is shown as total SLEDAI score.

b Absolute numbers of RAG+ and RAG- CD19+ IgD+ CD5+/− B cells in the peripheral blood of patient 1 before and during i.v. cyclophosphamide treatment were analyzed by a combination of lymphocyte cell count, FACS analysis and single cell PCR technique.
Figure 1

a  
![RAG1 and RAG2A/B (RAG+/beta-actin)](chart)

- **Frequency (%)**
  - **Pat. 1**: 0
  - **Pat. 2**: 5
  - **Pat. 3**: 10
  - **Healthy Individuals**: 20

- **p < 0.01**
- **p < 0.05**
- **n.s.**

b  

<table>
<thead>
<tr>
<th></th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocytes/ul</td>
<td>3400</td>
<td>4870</td>
<td>5060</td>
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<tr>
<td>RAG+CD5+/ul</td>
<td>11.1</td>
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<tr>
<td>RAG+CD5-/ul</td>
<td>50.4</td>
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</tbody>
</table>


c  

- **Total SLEDAI Score**
  - **Pat. 1**: 20
  - **Pat. 2**: 10
  - **Pat. 3**: 10
Figure 2

(a) VpreB+/beta-actin and CD154+/beta-actin expression levels in Patient 1 and Healthy Individuals. The bars indicate the frequency (%) of CD5+ and CD5- cells.

(b) VpreB+ RAG+/beta-actin and CD154+ RAG+/beta-actin expression levels in Patient 1 and Healthy Individuals. The bars indicate the frequency (%) of CD5+ and CD5- cells.
Figure 3

a

RAG+/beta-actin

Frequency (%)

0 5 10 15 20 25 30 35 40 45

before after 1 after 2 after 7 Healthy Individuals

i.v. cyclophosphamide treatments

b

CD19+IgD+CD5+

CD19+IgD+CD5-

Absolute cell count per µl

before after 1 after 2 after 7

i.v. cyclophosphamide treatments

SLEDAI

Total Score x100mg/d

Urinary protein

Proteinuria

before after 1 after 2 after 7 i.v. cyclophosphamide treatments