EXTENDED REPORT

Analysis of RAG expression by peripheral blood CD5+ and CD5- B cells of patients with childhood systemic lupus erythematosus

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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 is associated with the development of autoimmune diseases.

Objective: To investigate RAG expression in the CD5+ or CD5- IgD+ B cell compartment in childhood systemic lupus erythematosus (SLE).

Methods: Using a combination of flow cytometric cell sorting and reverse transcriptase polymerase chain reaction 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: While only one patient had a significantly increased frequency of RAG+ B cells in the CD5- B cell population, all patients showed higher frequencies of RAG+ B cells in the CD5+IgD+ B cell population. The frequency of RAG+ IgD+CD5+/ - B cells was reduced during intravenous cyclophosphamide treatment. In healthy age matched children, RAG expressing IgD+ B cells were hardly detectable. Coexpression of RAG and VpreB or CD154 mRNA could only be found in SLE B cells.

Conclusions: 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.

Systemic lupus erythematosus (SLE) is an autoimmune disease affecting both adults and children. Although childhood SLE resembles adult SLE in its presentation, clinical findings, and pathogenesis, children seem to have more severe disease at onset, with higher rates of organ involvement, and a more aggressive clinical course.¹ SLE is characterised by a broad range of abnormalities of the immune system and by multiorgan tissue pathology.²⁻³ High affinity autoantibodies to double stranded DNA (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) is, however, 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 (RAG) 1 and 2.⁶⁻⁷ 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 for preventing autoimmunity.¹¹⁻¹⁴

It has been shown that receptor editing in the bone marrow prohibits autoimmunity in transgenic animals and appears to be a powerful mechanism for protecting 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 centre 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 were able to show an increase in 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 seems rather 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 can 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 centre 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

Abbreviations: ANA, antinuclear antibody; BCR, B cell receptor; PBMC, peripheral blood mononuclear cell; RAG, recombination activating gene; SLE, systemic lupus erythematosus; SLEDAI, systemic lupus erythematosus disease activity index

expression in peripheral SLE B cells has been associated with CD154 mRNA expression.^{22–25}

B cells can be subdivided into two subpopulations with respect to their expression of CD5: B-1 B cells, which are mainly CD5+, and conventional B-2 B cells, which lack surface expression of CD5.³⁶ B-1 B cells are known to produce low affinity polyclonal antibodies, which recognise 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 remains unclear. Recently, increased expression of RAG was detected in peritoneal B cells of NZB mice, a murine model for human SLE.³⁹ This suggested that receptor revision outside the bone marrow could be an ongoing molecular process in B-1 B cells, which might 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 paediatric SLE patients.

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 three subjects with active SLE and two healthy donors. The subjects fulfilled the revised American College of Rheumatology (ACR) criteria for the classification of SLE.⁴ At the time of analysis, case 1 (male, 11 years) presented with type IV glomerulonephritis and thrombocytopenia; case 2 (female, 18 years) with thrombocytopenia; and case 3 (female, 13 years) with type II glomerulonephritis and a malar rash. All patients were antinuclear antibody (ANA) and anti-dsDNA antibody positive. Anti ds-DNA antibodies were measured using a radioimmunoassay (RIA), an enzyme linked immunosorbent assay (ELISA), and an immunofluorescence test. ANA titres were analysed using an immunofluorescence test.

PBMCs of case 1 were separated before and after one, two, and seven intravenous cyclophosphamide treatments. Ten treatments were given. In this patient, the absence of clinical symptoms and normalisation of laboratory values was achieved after one year (the seventh cyclophosphamide pulse). However, the anti-DNA and ANA titres did not change during and after discontinuation of 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. The healthy controls (10 year old female, 16 year old male) showed no sign of any 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 immunoglobulin (Ig) D and CD5 for 30 minutes, using anti-human CD19 antibodies (tricolour labelled, Caltag, Burlingame, California, USA), anti-human IgD antibodies (fluorescein isothiocyanate labelled; Caltag), and CD5 antibodies (phycoerythrin labelled; Caltag). Three colour immunofluorescence analysis was used for identification of the different B cell populations. Isotype matched antibodies were used as controls. FACS analysis was undertaken with a FACStar flow cytometer, using CellQuest software (Becton Dickinson, Franklin Lakes, New Jersey, USA).

Individual CD19+IgD+CD5+ and CD19+IgD+CD5– B cells of the SLE patients were sorted into 96-well PCR plates using a FACS Vantage flow cytometer (Becton Dickinson) outfitted with a single cell deposition unit, as described previously.^{21–22}

Preparation of RNA and cDNA from sorted single cells

Ten microlitres of lysis solution (2 µl of 5× first strand buffer (Invitrogen, Karlsruhe, Germany), 10 mM dithiothreitol (Invitrogen), 1% Nonidet-NP40 (Sigma, St Louis, Missouri, USA), 10 units of recombinant Rnasin ribonuclease inhibitor (Promega, Madison, Wisconsin, USA), 0.8 mM of each dATP, dCTP, dGTP, dTTP (Sigma), and 0.1 µg oligo d(T)_{12–18} (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA) 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.^{21–22} 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 reverse transcriptase were run in parallel and did not yield a product. RAG amplification from genomic DNA was excluded as described in previous experiments.^{21–22}

Table 1 Sequences of oligonucleotides used as primers for the amplification of cDNA or for the detection of polymerase chain reaction products by dot-blotting

Name	Sequence
β-Actin	
Sense	5′GTCCTCTCCCAAGTCCACACA 3′
Antisense	5′CTGGTCTCAAGTCAGTGTACAGGTAA 3′
Nested sense	5′TGATAGCATTGCTTCGTGTAA 3′
Nested antisense	5′TACATCTCAAGTTGGGGGACA 3′
Oligo	5′TTGAATGATGAGCCTTCGTG 3′
RAG1	
Sense	5′GAGCAAGGTACCTCAGCCAG 3′
Antisense	5′AACATGGCTGAGTTGGGAC 3′
Nested sense	5′TTCTGCCCCAGATGAAATTC 3′
Nested antisense	5′TGACCATCAGCCTGTCCAG 3′
Oligo	5′TCTCTGGAGCAATCTCCAGCA 3′
RAG2	
Exon 1A sense	5′GCAGCCCCTCTGCCTTC 3′
Exon 1B sense	5′GCGGTCTCCAGACAAAATC 3′
Antisense	5′TTTCAGACTCCAAGCTGCCT 3′
Nested sense	5′TCTCTGCAGATGGTAACAGTCAG 3′
Nested antisense	5′AGCGAAGAGGAGGGAGGTAG 3′
Oligo	5′TTCTTGATGTAAGCAT 3′
VpreB	
Sense	5′TGCACAGTGTGGTCTCAG 3′
Antisense	5′TCTCCCTCTCTCTCTCC 3′
Nested sense	5′AGTTGTGGTCTCAGCCG 3′
Nested antisense	5′GATGTCATGGTCTCTCA 3′
Oligo	5′CTTGAACCAATCCGC 3′
CD154 (CD40L)	
Sense	5′CACCATGAGCAACAACCTGGG 3′
Antisense	5′CTTGGCTTGGATCAGTCACA 3′
Nested sense	5′CCCTGGAAATGGGAAACAG 3′
Nested antisense	5′ACAAACACCGAAGCACCTG 3′
Oligo	5′TTATGAGGAGTGGGCAGGCTCAG 3′

Table 2 Total number of cells analysed (β -actin positive)

Individuals/samples	Number of cells analysed (β -actin positive)	
	CD19+IgD+CD5+	CD19+IgD+CD5-
Patient 1 before cyc	50	10
Patient 1 after 1 cyc	71	10
Patient 1 after 2 cyc	68	10
Patient 1 after 7 cyc	63	65
Patient 2	219	231
Patient 3	100	117
Healthy controls	121	118

cyc, intravenous cyclophosphamide treatment.

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 hybridisation buffer containing DIG-dUTP labelled 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. Visualisation was obtained by exposure to a photographic film. The blots were analysed on a digital detection unit (Biorad, Hercules, California, USA) using QuantityOne Software (Biorad). Only β -actin positive cells were considered for the analysis of RAG1 and 2, VpreB, or CD154 mRNA expression. The number of the cells analysed in each patient is shown in table 2.

Statistical analysis

Statistical analysis for comparison of single cell gene expression patterns of different B cell populations was undertaken using the χ^2 test.

RESULTS

Coordinated expression of RAG1 and RAG2 mRNA in SLE

As 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. IgD+ B cells seem to be the population with the highest frequencies of RAG+ cells in SLE,²² so we were interested to characterise 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% (fig 1), whereas no coordinated expression of RAG1/2 mRNA could be found in the CD5+ population of the two healthy controls. RAG 1/2 mRNA expression was upregulated in peripheral IgD+ B cells of SLE patients (range 0.9% to 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 CD5- population of SLE patients with their counterpart in the healthy age matched controls, significant differences were obvious (fig 1A). All three SLE patients showed significantly higher frequencies of RAG+ cells in the CD5+ B cell population than the CD5+ B cells of the healthy controls. However, in the CD5- B cell population only one patient showed increased frequencies of RAG+ cells when compared with the healthy individuals (fig 1A).

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

Figure 1 RAG1 and RAG2A/B mRNA expression in CD5+ and CD5- IgD+ peripheral blood B cells of patients with systemic lupus erythematosus at the time of diagnosis. While only one patient showed a significantly higher frequency of RAG+ B cells in the CD5- B cell population, all patients had 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 the three SLE patients and the healthy controls (mean of two) are shown. Frequencies (A) of RAG+ cells were compared with the corresponding B cell population in the healthy controls. Absolute cell counts of RAG+ cells are shown in (B). Only β -actin mRNA positive cells were considered for the analysis of the frequency of RAG1 and RAG 2 mRNA expressing cells. Frequencies were calculated as (RAG1+ RAG2A/B+)/(β -actin) $\times$ 100. 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). RAG, recombination activating gene; SLEDAI, systemic lupus erythematosus disease activity index.

patient with highest disease activity (type IV glomerulonephritis, thrombocytopenia, SLEDAI total score of 21; Patient 1) (fig 1B).

Frequency of CD154 or VpreB mRNA expressing cells in CD5+ and CD5- IgD+ SLE B cells

To analyse whether 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 case 1, who showed highest disease activity.

Healthy controls showed a moderate frequency of VpreB mRNA expressing cells in both the CD5+ and CD5- B cell population (5.8% and 4.2% respectively) (fig 2A). The

Figure 2 VpreB, CD154 (CD40Ligand), and RAG1/2 mRNA expression in peripheral blood systemic lupus erythematosus B cells at the 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+/- SLE B cells showed 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 controls (mean of two) and case 1 was achieved. (A) The frequency of cells indicating VpreB mRNA expression was calculated as (VpreB+/ β -actin) $\times$ 100. 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+/ β -actin) $\times$ 100. The frequency of CD154+ and RAG+ B cells was calculated in the same way. RAG, recombination activating gene.

frequency of VpreB+ B cells was significantly increased in both CD5+ and CD5- B cells of this SLE patient as compared with the healthy controls (18% ($p < 0.05$) and 30% ($p < 0.01$), respectively). However, neither a significant difference in the frequency of VpreB+ cells between the CD5+ and the CD5- population in the SLE patient ($p > 0.05$), nor a characteristically dominant increase in VpreB mRNA expression in any particular one of these populations compared with the healthy controls could be shown.

Similar patterns were analysed for the expression of CD154 mRNA (fig 2A). The frequency of CD154 mRNA expressing cells was significantly higher in both CD5+ and CD5- B cells of this SLE patient (24% ($p < 0.01$) and 20% ($p < 0.05$), respectively) compared with the healthy controls (3.3% and 4.2% respectively). No differences in the expression of CD154 mRNA could be found between the two cell populations analysed ($p > 0.05$).

IgD+CD5+/- SLE B cell 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 case 1 in both the CD5+ B cell population (4%) and the CD5- B cell population (10%). However, statistical analysis did not

achieve significance when expression frequencies were compared in between CD5+ and CD5- B cells ($p > 0.05$). Of note, they were not detectable in the healthy controls (0%, 0%) (fig 2B).

Whereas 2% of all CD5+ and 10% of all CD5- peripheral IgD+ B cells in SLE case 1 coexpressed RAG1/2 and CD154 mRNA, no coexpression of these markers could be found in these cell populations of the healthy controls. The differences between CD5+ and CD5- B cells in the patient did not achieve significance ($p > 0.05$) (fig 2B).

CD5+ RAG+ B cells and 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 intravenous 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 seventh intravenous cyclophosphamide treatment (fig 3A). Notably, the frequency of CD154+ or VpreB+ cells in the RAG+ B cell population was also reduced after one cyclophosphamide treatment in both CD5+ and CD5- CD19+ IgD+ B cells, in contrast to those cells expressing only CD154 or VpreB mRNA but not RAG1/2 mRNA (data not shown). Both proteinuria and the SLEDAI score decreased in this patient after the first cyclophosphamide treatment, and absence of clinical symptoms was achieved after the seventh treatment (fig 3A). Interestingly, after the seventh cyclophosphamide treatment the IgD+ B cell population was dominated mainly by CD5- B cells which did not express RAG1/2 and CD154 or VpreB (fig 3B).

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 the control of several central B cell tolerance mechanisms, thereby avoiding the generation 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 in the BCR repertoire towards autoreactivity.

In this study we analysed 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 controls. In the healthy controls, 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 from a previous study, showing peripheral RAG expression in adolescent healthy mice only in the CD5- and not in the CD5+ B cell population.⁴⁰ Interestingly, whereas only one patient in this study had significantly higher frequencies of RAG+ B cells in the CD5- B cell population, all three patients showed significantly higher frequencies of RAG+ B cells in the CD5+ population. Thus one might conclude that RAG expression seems to be upregulated in the CD5+ B cell population in particular in SLE patients. This is supported by experiments analysing RAG expression in murine lupus.³⁹

Figure 3 RAG1/2 mRNA expression in peripheral blood systemic lupus erythematosus B cells at time of diagnosis and during intravenous cyclophosphamide treatment. The frequency of CD5+RAG+ B cells decreased during the 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+ B cells (black) and CD5- B cells (white) of healthy controls (mean of two) and case 1 before and after one, two, and seven cyclophosphamide pulses. (A) Frequencies were calculated as $(RAG1+RAG2/\beta\text{-actin}) \times 100$. The disease activity of case 1 during intravenous 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 case 1 before and during intravenous cyclophosphamide treatment were analysed by a combination of lymphocyte cell count, FACS analysis, and single cell PCR technique. FACS, fluorescence activated cell sorting; PCR, polymerase chain reaction; RAG, recombination activating gene; SLEDAI, systemic lupus erythematosus disease activity index.

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 the 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 autoreactive or polyreactive BCRs.³⁶ As clearance of apoptotic cells is impaired in SLE, huge amounts of autoantigens might be presented in this context.⁴¹ Polyreactive or autoreactive CD5+ B cells might recognise 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 in fact B cell precursors recently emigrated from the bone marrow and still expressing some 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 in 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 has 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}

Nevertheless, observing RAG expression in IgD+ but not in IgG+ SLE B cells,²² as well as the fact that not all the RAG+ B cells in this study coexpress CD27 mRNA (data not shown), assigns these RAG expressing B cells to an immature or transitional stage rather than to the memory B cell compartment. Interestingly, some of these RAG expressing cells in our study showed signs of activation by the coexpression of CD154 mRNA (fig 2). Coexpression 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 that 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 were able to demonstrate increased frequencies of RAG expressing B cells, particularly in the IgD+CD5+ B cell population, which might be evidence for ongoing V(D)J recombination in these cells.^{22, 44} Uncontrolled V(D)J recombination outside the bone marrow might account for the peripheral generation of high affinity autoreactive B cells in SLE.

In the current analysis we were able to show that both IgD+ CD5+ and CD5- B cell populations were markedly reduced after two treatments with intravenous cyclophosphamide (fig 3). In addition, 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 seventh cyclophosphamide treatment, when absence of clinical symptoms

was achieved. The dominating CD5[−] B cell subset did not show RAG expression. This finding is an argument for the 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 in case 1. Additionally, despite 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 cyclophosphamide treatments. This raises the question of whether RAG expression in peripheral blood B cells in patients with high disease activity (for example, case 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 can be shown that both CD5⁺ and CD5[−] B cells are capable of producing anti-dsDNA antibodies.^{45 46}

Conclusions

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.

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