ORIGINAL INVESTIGATIONS

Effect of biological agents on B-lymphocyte subpopulations in patients with systemic lupus erythematosus

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Objective: *to evaluate the effect of different biological agents (BAs), including rituximab (RTM) and belimumab (BLM) combination therapy, on B-lymphocyte subpopulations during a follow-up of patients with systemic lupus erythematosus (SLE).*

Patients and methods. *The investigation enrolled 64 patients with a verified diagnosis of SLE with high and moderate disease activities according to the Systemic Lupus Erythromatosus Disease Activity Index (SLEDAI)-2K scores; 47 patients of them took RTM, 10 used BLM, and 7 received RTM + BLM combination therapy. Peripheral blood B-lymphocyte subpopulations were measured by multicolor flow cytofluorometry, using a panel of monoclonal antibodies to B-lymphocyte surface membrane markers. The results were assessed using the SLEDAI-2K scores and the British Isles Lupus Assessment Group (BILAG) index.*

Results and discussion. *RTM therapy led to a marked decrease in major B-lymphocyte populations, the residual cells being naXve B-cells and different memory B cell populations, the percentage of which depended on the degree of depletion after a RTM cycle. Incomplete B-lymphocyte depletion was associated with the large baseline numbers of plasma cells (PCs) (>0.2%). One year after initiation of therapy, the percentage ratio of B-lymphocyte subpopulations returned almost completely to baseline values, except the whole memory B-cell population. BLM therapy resulted in a decrease in PCs and plasmablasts (PBs) to the point of their complete depletion. There were reductions in total CD19+ B-lymphocytes and naive B lymphocytes. The use of the combination of BAs permitted the monitoring of the total B-lymphocyte population; its slower recovery was seen in patients with its complete depletion after a rituximab cycle. The therapy promoted maintenance of low concentrations of PCs and PBs, total memory B-cell and naive B-cell populations.*

Conclusion. *In patients with SLE, all the three therapy with BAs demonstrated a good efficiency manifested by a decrease in clinical and laboratory disease activity. The found time course of changes in B-lymphocyte subpopulations can be used for the selection of therapy and for the evaluation of its efficacy.*

Keywords: *systemic lupus erythematosus; B-lymphocyte subpopulations; rituximab; belimumab.*

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Systemic lupus erythematosus (SLE) is a multisystem autoimmune disease of unknown etiology characterized by the overproduction of organ-specific autoantibodies against different components of the cell nucleus with the development of immune-mediated inflammatory damage to tissues and viscera [1]. The leading role in the pathogenesis of the disease is played by autoreactive B-lymphocytes that synthesize autoantibodies against a variety of antigens, participate in the synthesis of cytokines and the presentation of the antigen to T lymphocytes [2]. The generation of B lymphocytes includes several stages: from pro-B-cells to a mature B cell. Contact with the antigen subsequently leads to the formation of memory B cells and plasma cells (PCs) that are divided into short-lived and long-lived ones. In the development of a B cell, on its surface there are CD-membrane markers and immunoglobulins of different classes, which allows them to be identified and classified [3–5].

It is not surprising that the increased efficacy of treatment for SLE is associated with the clinical introduction of biological agents (BAs), such as rituximab (RTX) and belimumab (BLM), focused on the suppression of B lymphocytes. RTX is a chimeric monoclonal antibody to CD20, which causes B-cell depletion. Belimumab (BLM), a human recombinant monoclonal antibody (IgG1λ) that prevents the interaction of pBLyS (a B-lymphocyte stimulator) with autoreactive B-lymphocyte cell receptors, thereby reducing B-cell hyperreactivity and survival of autoreactive B-lymphocyte clones, was registered in 2010 [6]. Currently, the cell markers for the efficiency of therapy with BAs are the degree of B-cell depletion and the time course of changes in the clinical and laboratory signs of disease activity; and the markers for an exacerbation are the repopulation or preservation of residual B lymphocytes after the use of BAs [7–9].

Nevertheless, when using the same treatment regimens in patients with the similar clinical course of the disease, the effect and remission cannot always be achieved [8]. There is still a problem of identifying indicators that would characterize the prognosis of SLE, the outcomes and efficiency of treatment in a particular patient; there are no reliable biomarkers of lupus for diagnosis, monitoring, and prediction of response to therapy. The search for clinical and laboratory indicators that contribute to the prediction of a future therapy response, relapse and course of the disease is relevant. In this regard, interest in the study of specific components in the pathogenesis of the disease is natural. Since a huge role in the development of SLE is played by B cells; and current methods of therapy are focused on their suppression; to define the role of individual B-lymphocyte subpopulations in patients is important in determining the prognosis of the disease. Thus, a detailed analysis of B-lymphocyte subpopulations can contribute to the solution of the tasks set. There are scarce data about the original composition of B lymphocyte the subpopulations in patients with SLE and their changes during treatment with BAs, but the material accumulated by investigators makes it possible to identify them as an important tool for evaluating the course of SLE and assessing the outcomes of therapy.

Brief characteristics of B lymphocyte subpopulations

Memory B cells are subdivided into pre-switch IgD+/IgM+ and post-switch IgD-/IgG+, IgA+. Stimulation of these cells occurs in a reduced way (they have lower activation thresholds and a hyper-response to various stimuli), and, consequently, leads to the further production of PCs secreting autoantibodies [10]. Memory B lymphocytes can represent antigens due to the expression of B-cell receptors and major histocompatibility complex class II molecules [3, 11].

Double negative (DN) (CD27-, IgD-) B lymphocytes are characterized as activated memory B cells. Their origin has not fully been studied, but according to available data, these cells can play a substantial role in the development of SLE. The increase in their number correlates with the development of SLE, and they often account for a significant part of all memory B memory cells [10, 12].

PCs are classified into long-lived (CD19+, CD138+) and short-lived (CD19+, CD38+). The peculiarity of these cells is that their surface lacks the CD20 receptor that serves as a main target for RTX. After activation, some part of naXve B-lymphocytes is differentiated into short-lived PCs (CD19+, CD38high+, CD20-, CD27high) that secrete mainly anti-double-stranded DNA (anti-dsDNA) antibodies in SLE [3, 8]. The rest of the naXve B-cells develop in the germinal centers. The end result is the production of memory cells and long-lived PCs that secrete antibodies against Ro, La, and Sm antigens [3].

BLyS (BAFF) and APRIL regulate activation and differentiation and increase the survival of auto-reactive B-cell clones. They have three receptors: 1) BAFF-R, 2) transmembrane activator, calcium modulator and cyclophilin ligand interactor (TACI), and 3) B-cell maturation antigen (BCMA), each of which is differently expressed by B cells during their ontogenesis. BAFF-R is expressed by all mature and memory B cells, is suppressed in the germinal centers, and appears on the PCs. BCMA is mainly present on the plasmablasts (PBs), but is also detected on a small number of DN B cells. TACI is expressed by B lymphocytes, PBs, and PCs [13].

Objective: to evaluate the effect of different BAs, including RTM and BLM combination therapy, on B-lymphocyte subpopulations during a follow-up of patients with SLE.

Patients and methods. The investigation enrolled 64 patients (with 59 (92%) women and 5 (6%) men) with a verified diagnosis of SLE with high and moderate disease activities according to the SLEDAI-2K. The median (Me) age was 33 [25; 40] years for the whole group. Forty-seven patients were treated with RTM at a dose of 500 to 2000 mg. During repeated visits (at 3, 6, and 9 months), 21 patients underwent planned administration of RTX at a dose of 500–1000 mg. Ten patients were prescribed BLM at a dose of 10 mg/kg body weight every month. BLM was used in 10 patients with predominantly mucocutaneous, articular lesions who had hematologic disorders. Combined therapy with RTX+BLM was prescribed to 7 more patients. They were injected with RTX 500–1000 mg; and 3 months later, BLM was prescribed by the standard scheme of 10 mg/kg once monthly for 8 months. There were no repeated RTX cycles in this group during the follow-up period (Tables 1 and 2).

All patients received standard therapy including immunosuppressive drugs, glucocorticoids (GCs), and, in the use of BAs, 55 (85%) patients underwent pulse therapy with 6-methylprednisolone at a dose of 0.25 to 3 g. In general, in the group of patients treated with RTX, the dose of GCs was 15 [10; 28] mg/day. The patients on BLM used medium and low GC doses (15 [5; 20] mg/day). During RTX + BLM combination therapy, the patients also received medium and low doses of GCs of 20 to 5 (10 [5; 15] mg/day). Due to the involvement of vital organs, some patients took cytostatic drugs, including cyclophosphane (as a short-term cycle), mycophenolate mofetil, and methotrexate.

At the time of inclusion and every 3 months during a year, all the patients underwent a standard examination adopted for SLE: clinical and biochemical blood tests, urinalysis, and immunological examination (determination of anti-dsDNA, C3c and C4 complement components, IgG, IgA, and IgM). If necessary, chest radiography, abdominal ultrasound, and echocardiography were performed. SLEDAI-2K and BILAG scores were estimated over time. Peripheral blood B-lymphocyte subpopulations were measured by multicolor flow cytometry using a panel of monoclonal antibodies to B-lymphocyte surface membrane markers. The results of four-color staining of B-lymphocytes were assessed using a Beckman Coulter NAVIOS™ flow cytometer (Beckman Coulter, USA). A total of 50,000 events were counted for each analysis. B-cell populations were identified by the CXP software (Beckman Coulter, USA). When gating on the horizontal and vertical axes, the percentage of lymphocytes (CD45+) and B cells (CD19+) was determined; and the expression of the surface membrane markers IgD, CD20, CD27, CD38, CD10, and CD138 was used to quantify B cell subpopulations (Table. 3).

B-cell depletion of CD19+ B lymphocytes was evaluated at 3 months of a RTX cycle: complete depletion (0%); partial depletion (0.1 to 0.5%); no depletion (>0.5%). Repopulation of B-lymphocytes was considered to be their recovery (>1%) in patients who had achieved complete or partial deletions.

The results were statistically processed using Statistica 7.0 (StatSoft, USA), including nonparametric methods. For the parameters, the distribution of which differed from the normal one, the Mann–Whitney U-test was used to compare the two groups, the results are presented as a median (Me) [25th; 75th percentile]. Descriptive statistics were also applied. Statistical significance was defined as $p<0.05$.

Table 1. Characteristics of patients included in the study

	RTX (n=47)	BLM (n=10)	RTX+BLM (n=7)
Age, years	35 [25; 40]	32 [26; 34]	21 [20; 29]
female/male, n	44/3	9/1	6/1
Disease duration, n:			
0-5 years	30	5	4
>6 years	17	5	3
SLEDAI-2K, scores:	16 [11; 20]	10 [8; 11]	10 [9; 16]
SLEDAI-2K, n:	-		
grade 2 activity	10	6	3
grade 3 activity	37	4	4
BILAG total	18 [14; 25]	16 [12; 17],	17 [10; 18].
Involvement of organs and systems, n (%):	-		
LN	19 (40)	0	1 (14.2)
neurolupus	8	0	0
vasculitis	5 (10,6)	0	2 (28.6)
skin	19 (40)	7 (70)	3 (42)
mucosae	15 (32)	3 (42)	4 (57)
arthritis	21 (44.7)	6 (60)	4 (57)
serositis	18 (38)	3 (30)	1 (14.2)
hematologic disorders	27 (57)	5 (50)	5 (71)
SLICC/DI ≥1, n (%)	20 (42.5)	2 (28.6)	4 (57)

Note. Where not otherwise specified, the data are presented as Me [25; 75]. LN - lupus nephritis; DI - damage index.

Results (the authors' findings)

Baseline numbers of B-lymphocyte subpopulations in SLE patients and healthy donors

Estimation of total counts of CD19+ B-lymphocytes at baseline revealed no significant differences in 64 patients with SLE and in healthy donors. Table 4 shows the absolute and percentage values of different B-lymphocyte subpopulations in 64 SLE patients and in 20 healthy donors at the time of inclusion in the study. Patients with SLE had low numbers of switched memory B cells, while the counts of DN B lymphocytes and PBs were higher.

Impact of RTX treatment on B-lymphocyte subpopulations

Time course of changes in B lymphocytes at 3 months after administration of BAs. The time course of changes in B lymphocytes during this period was evaluated in 54 patients who received RTX (n=47) and RTX and BLM combination therapy (n=7). The degree of B-cell depletion by at 3 months did not depend on

Table 2. Initial and repeated visits, n

Visit	RTX	BLM	RTX + BLM	Total
At Initial visit	47	10	7	64
At 3 months	47	10	7	64
At 6 months	42	7	7	56
At 9 months	31	5	7	43
At 12 months	34	5	5	44

Table 3. B-lymphocyte subpopulations and molecular markers

B lymphocytes	Molecular markers
Naïve B cells	CD19+, CD27–, IgD+
Total memory B cell population	CD19+, CD27+
Unswitched memory B cells	CD19+, CD27+, IgD+
Switched memory B cells	CD19+, CD27+, IgD–
PBs	CD19+, CD38+++, CD27+, IgD–, CD20–
PCs	CD19+, CD38+
DN memory B cells	CD19+, CD27–, IgD–

Note: PBs – plasmablasts; PCs – plasma cells; DN – double-negative.

Table 4. B-lymphocyte subpopulations in SLE patients and healthy donors (Me [25; 75])

B lymphocytes	Molecular markers	Number of B-cells, % / ($\times 10^9$/l)		p
		Patients with SLE (n=64)	Healthy donors (n=20)	
Total population of B lymphocytes	CD19+ B cells	8.85 [4.4; 13.5] / 0/1 [0.08; 0.25]	8.5 [7.2; 11.0] / 0.2 [0,09; 0,2]	0.79 / 0.56
Naïve B cells	CD19+, CD27–, IgD+	67 [49; 78] / 0.08 [0.03; 0.13]	64.7 [57.6; 72.4] / 0.1 [0.06; 0.1]	0.15 / 0.44
Memory B cells (total population)	CD19+, CD27+	1 [0.6; 1.8] / 0.001 [0.0006; 0.004]	2.2 [1.1; 3.0] / 0.003 [0.001; 0.007]	0.028 / 0.12
Unswitched memory B cells	CD19+, CD27+, IgD+	5 [2.7; 8.2] / 0.005 [0.0026; 0.01]	7.4 [3.7; 11.1] / 0.01 [0.005; 0.02]	0.12 / 0.056
Switched memory B cells	CD19+, CD27+, IgD–	4.85 [2; 11] / 0.0079 [0.003; 0.015]	12.8 [9.3; 17.0] / 0.02 [0.01; 0.04]	0.001 / 0.0025
PBs	CD19+, CD38+++, CD27+, IgD–, CD20–	0.3 [0.1; 0.4] / 0.0003 [0.00017; 0.00077]	0.1 [0.1; 0,2] / 0.0002 [0.0001; 0.0004]	0.022 / 0.33
PCs	CD19+, CD38+	0.1 [0.1; 0.2] / 0.0001 [0.00005; 0.00038]	0.1 [0.05; 0.1] / 0.0001 [0.00; 0.0002]	0.06 / 0.2
DN memory B cells	CD19+, CD27–, IgD–	18 [11.1; 29.6] / 0.02 [0.01; 0.04]	13.3 [7.1; 19.3] / 0.02 [0.01; 0.02]	0.02 / 0.19

Note: PBs – plasmablasts; PCs – plasma cells; DN – double-negative.

Table 5. Degree of B-lymphocyte depletion at 3-month follow-up, n (%)

RTX, mg	complete depletion	partial depletion	no depletion
500 (n=12)	4 (33)	5 (42)	3 (25)
1000 (n=39)	21(54)	9 (23)	9 (23)
2000 (n=3)	2 (67)	–	1 (33)
Total (n=54)	27 (50)	14 (26)	13 (24)

the baseline SLE activity (SLEDAI-2K) and duration of disease. At 3 months, complete B-lymphocyte depletion was more common in patients taking higher RTX doses; complete depletion could be achieved in 50% of the patients (table. 5).

The SLEDAI-2K/BILAG disease activity at baseline was 14 [10; 18]/17 [13; 22] scores, at 3-month follow-up it was noted to decrease to 4 [2; 10]/8 [1; 10] scores in patients with complete depletion, to 5 [4; 8]/8 [1; 9] scores in those with partial depletion and to 8 [5; 10]/9 [2; 16] scores in those with no depletion ($p<0.00000$). The concentration of anti-dsDNA antibodies reduced to 54 [14; 111], 45 [25; 75], and 135 [33; 300] U/ml in patients with partial, complete, and no depletion, respectively ($p<0.000003$). However, the levels of C3c and C4 complement component were much higher in patients with complete depletion than in those with no depletion: C3c, 0.97 [0,84; 1,18], 0,75 [0,66; 0,97], and 0.69 [0.52; 0.78] g/l; C4, 0.17 [0.13; 0.21], 0.13 [0.12; 0.15], and 0.13 [0.072; 0.16] g/l, respectively ($p<0.0007$ and $p<0.02$ for patients with complete depletion and for those with partial depletion).

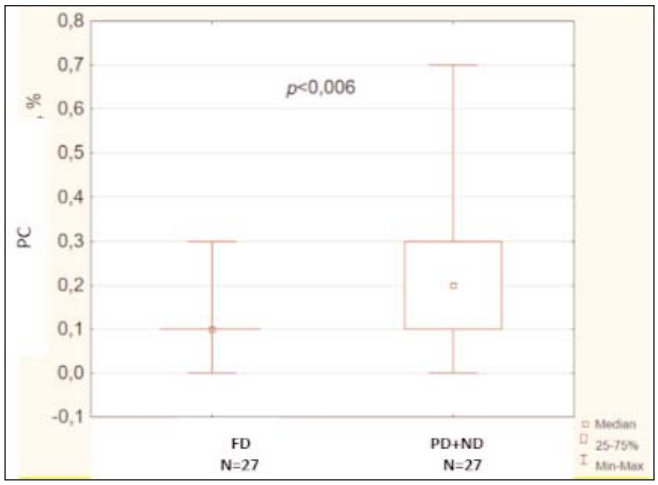

Fig. 1. *Baseline content (%) of PCs (CD19+, CD38+): patients with complete depletion (CD) at 3 months after RTX cycle (n=27); patients with partial deletion (PD) and no depletion (ND) after a RTX cycle (n=27)*

Fourteen patients with partial depletion who responded to treatment were observed to have lower levels of all B-cell subpopulations at 3 months after the first RTX cycle; therapy led to a rapid depletion of naXve and DN memory B cells. The residual cells were predominantly naXve, unswitched, switched, and DN memory B cells. A special feature was their percentage redistribution with a tendency to increase the proportion of memory B cells: DN (27.7 [18.4; 50]%), switched (22.7 [4.3; 50]%), unswitched (20 [2.5; 35]%) memory B cells with a relatively lower number of naXve B cells (15 [8.7; 23.3]%), moreover, one half of the patients had predominantly the proportion of unswitched memory B cells; the other half had switched ones. The percentage of the total population of memory B cells was significantly reduced (0.1 [0; 0.2]%; p<0.000007).

Thirteen patients with no depletion also showed a decrease in the total population of CD19+ B lymphocytes versus the baseline levels; however, their percentage did not practically differ from the baseline one, i.e. the numbers of naive (30 [16; 60]%) and DN (42 [20; 51]%) memory B cells remained high with the lower values of switched (6.6 [2.2; 23]%) and unswitched (4.5 [2.8; 10.8]%) memory B cells.

Patients with a higher baseline percentage of PCs were more frequently found to have partial depletion of the total population of CD19+ B lymphocytes at 3 months after initiation of therapy with BAs (p<0.006; Fig. 1). Thus, in 17 (62%) of the 27 patients with partial depletion and no depletion of B lymphocytes, the level of PCs was equal to or greater than 0.2% (with the maximum value of up to 0.7%), whereas in 22 (81%) of the 27 patients with complete depletion, the numbers of PCs were 0–0.1% (p<0.006).

Time course of changes in B lymphocytes within 6 to 12 months after administration of BAs. At 3-month follow-up, 38 (78%) patients showed repopulation in more due to naXve, DN, unswitched, and switched memory B cells.

After a RTX cycle and subsequent recovery of CD19+ B-lymphocytes at 6 months, 16 patients with complete depletion displayed a slower repopulation of the total group of memory B cells (0.1 [0.1; 0.2]%) and naXve B cells (32 [17; 68]%). However, half of them were recorded to have a higher percentage of unswitched memory B cells (17 to 83%), and the other half had switched ones (14 to 87%).

To evaluate the effect of RTX on B-lymphocyte subpopulations, 16 patients who received RTX once were identified during the long-term follow-up, i.e. repeated cycles of BAs were not performed in the subsequent period. In these patients, the percentage of almost all B-lymphocyte subpopulations recovered at 12 months; however, when compared with the baseline distribution, they were found to have a significantly lower percentage of the total population of memory B cells (0.15 [0.1; 0, 3]; p<0.000012).

Impact of BLM treatment on B-lymphocyte subpopulations

Ten patients with moderate and high disease activity took BLM; 3 of them used the drug for 9 (7–12) months (due to its absence in the pharmacy network). During BLM therapy, clinical and immunological responses were recorded in 9 patients. This treatment led to a gradual SLE activity decrease, which was recorded at 3-month follow-up in 7 patients, at 6 and 9 months in 2 more patients: SLEDAI-2K/BILAG at baseline (10 [8; 11]/16 [12; 17] scores), after 12 months (4 [2; 4]/1 [1; 5] scores). Immunological blood tests revealed a decrease in the anti-dsDNA antibody titers: 113 [73; 300] U/ml at baseline, 73 [27; 206] U/ml at 12 months and an increase in the level of the components of complement C3: 0.7 [0.57; 0.9] g/l at baseline, 0.9 [0.7; 0.94] g/l at 12 months and C4: 0.09 [0.06; 0.12] g/l at baseline, 0.15 [0.12; 0.2] g/l at 12 months. The changes in these indicators were most pronounced at 6-month follow-up.

During 12 months of the study, the patients exhibited a decrease in the total population of CD19+ B lymphocytes (from 7.5 [6.4; 11.6] to 2.9 [1.5; 6.5]%); however, complete depletion was not achieved. There was a redistribution of the percentage of B-lymphocyte subpopulations towards reducing the proportion of naXve B-lymphocytes and increasing the number of unswitched and to a lesser extent DN memory B cells. At 3 months after initiation of BLM therapy, there was simultaneously a progressive decrease in the absolute values of naXve B lymphocytes and an increase in unswitched memory B cells. However, the number of the latter did not increase at 3 months. The drug did not substantially affect the population of the entire group of memory B cells (Table. 6).

Estimation of changes in PCs and PBs, there was a decrease in their absolute values at 6-9 months of BLM therapy; at 6-months follow-up, peripheral blood PBs were not determined in 4 of the 7 patients; peripheral blood PCs were absent in 3 of the 7 patients. However, the proportion of PCs increased at 12 months (Figs. 2 a, b).

Impact of RTX + BLM combination therapy on B-lymphocyte subpopulations

Seven patients with different symptoms of SLE mainly with high disease activity received the combination therapy. At 3 months after initiation of RTX therapy, its response was achieved in 4 patients. Two more patients achieved the response at 6-month follow-up after initiation of BLM therapy. The therapy was unsuccessful in one patient.

Assessment of SLE activity showed its progressive decrease according to SLEDAI-2K/BILAG scores during the follow-up period: 10 [9; 16]/17 [10; 18] at baseline and 3 [2; 6]/1 [1; 2] at 12 months. During the above period, the anti-dsDNA antibody titer decreased (174 [36; 300] and 12 [6; 34] U/ml at baseline and at

Table 6. Changes in B-lymphocyte subpopulations during BLM therapy

Period	Unswitched memory B cells		DN memory B cells		Naïve B lymphocytes	
	%	abs. ($\times10^9$/l)	%	abs. ($\times10^9$/l)	%	abs. ($\times10^9$/l)
at baseline (n=10)	3.5 [1.3; 6.8]	0.004 [0.002; 0.005]	15.2 [10.4; 22]	0.01 [0.01; 0.04]	70.4 [67; 83]	0.09 [0.05; 0.2]
at 3 months (n=10)	22.3 [9.9; 29.5]*	0.009 [0.008; 0.034]*	22 [17; 25.5]	0.01 [0.0095; 0.03]	45.5 [33; 63.8]	0.04 [0.02; 0.01]
at 6 months (n=7)	14 [7.3; 41]	0.009 [0.002; 0.03]	32 [18; 42]	0.02 [0.005; 0.046]	31.8 [22.7; 47.7]	0.02 [0.004; 0/04]
at 9 months (n=5)	18 [15.7; 22]	0.01 [0.004; 0.04]	28 [27.3; 32]	0.03 [0.02; 0.05]	22.7 [21.6; 48.8]	0.02 [0.02; 0.03]
at 12 months (n=5)	27.9 [24; 36.8]	0.01 [0.0065; 0.045]	39 [23; 46]	0.015 [0.0065; 0.03]	16.4 [14.3; 27.5]	0.013 [0.003; 0.04]

*p<0.008.

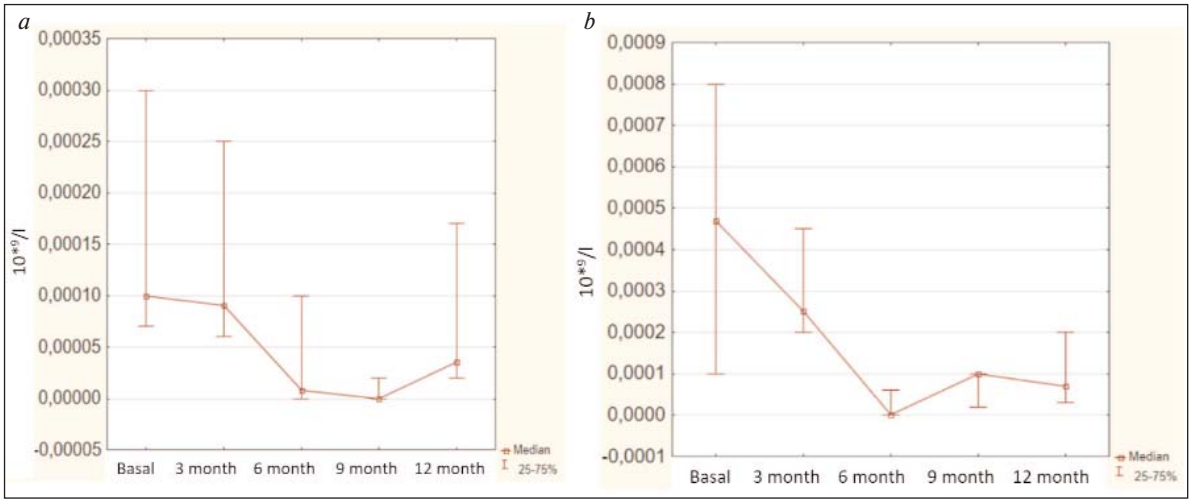

Fig. 2. *Changes in the absolute values ($\times10^9$/l) of B-lymphocytes subpopulations during BLM therapy during 12 months of follow-up: a – PCs; b – PBs*

12 months, respectively). At the same time, there was an increase in the content of the components of complement C3 (0.4 [0.37; 0.44] and 0.85 [0.81; 0.88] g/l at baseline and at 12 months) and C4 (0.038 [0.03; 0.054] and 0.17 [0.15; 0.26] g/l at baseline and at 12 months, respectively).

At 3 months, complete and partial depletions were achieved in 2 and 1 patients, respectively; no depletion was noted in 4 (57%) patients. Considering the previous findings of the effect of BLM on B lymphocytes, it seemed curious to estimate the time course of changes in the total population of B lymphocytes after addition of this drug to the therapy. For this purpose, the total population of B-lymphocytes was quantified in 16 SLE patients who used only RTM once; the results obtained were compared with the data of each patient who received combination therapy. It was found that after achieving complete and partial depletion, 16 patients on monotherapy with BAs showed a further increase in the level of B lymphocytes ($p < 0.003$), whereas the number of B lymphocytes continued to decline throughout the follow-up period in the patients receiving combination treatment with BAs (even despite the absence of complete depletion in a larger number of cases). In addition, patients with complete and partial depletion had a slower repopulation of B lymphocytes (Fig. 3a).

The level of PCs and PBs tended to increase in patients who took RTX once at 6 months. Combination therapy contributed to the maintenance of a low number of these cells in patients with complete and partial depletion or to the further reduction of their content in patients who did not achieved depletion after a RTX cycle (Fig. 3b).

B-lymphocyte repopulation occurred during combination therapy as during RTM monotherapy. At 12 months, the percentage of B-lymphocyte subpopulations almost corresponded to that at baseline; however, there was a higher proportion of unswitched memory B cells (3.7 [2.7; 14.5] and 23.6 [10.5; 39, 4]% at baseline and at 12 months, respectively) and a reduction in the number of naXve B cells (69 [21; 83] and 34 [10; 43]% at baseline and at 12 months, respectively) compared with the baseline composition. The percentage of the total population of memory B lymphocytes who had achieved an effect after RTM treatment also tended to decrease.

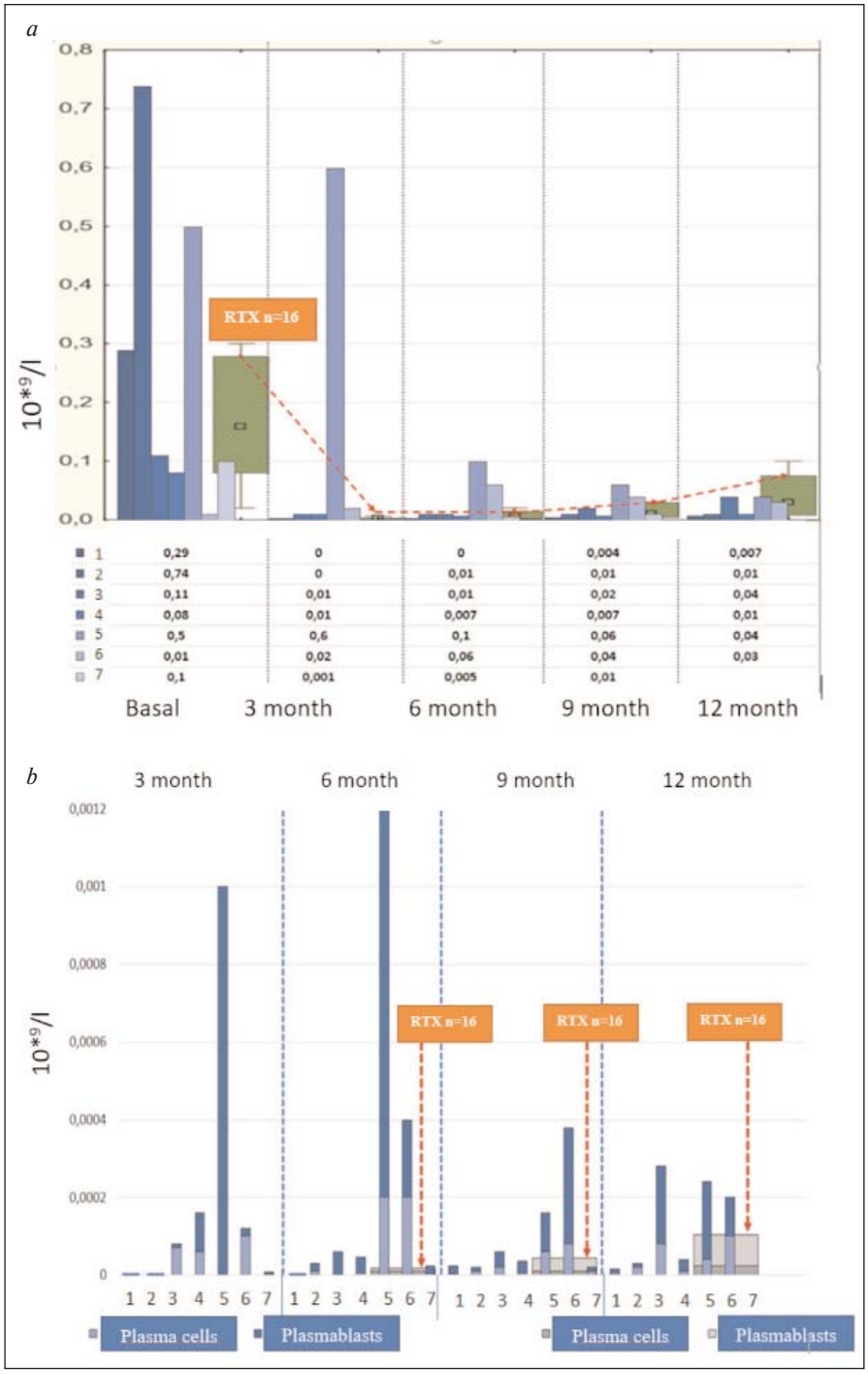

Fig. 3. *Time course of changes in the absolute content (_109/l) of CD19+ lymphocytes during combination therapy and RTX treatment: the blue scale indicates the number of CD19+ B lymphocytes in 7 patients on RTX + BLM combination therapy; the green scale indicates the number of CD19+ B lymphocytes in 16 patients who took RTX (a). The number (×10 ^{9}/l) of PBs and PCs in 7 patients who received combination therapy at 3, 6, 9, and 12 months (b)*

Discussion. B lymphocytes are a main goal of targeted treatment for SLE [14]. Several open-label experimental clinical studies have shown that B-cell-depleting RTM therapy is safe and effective in treating SLE, including LN [15]. BLM, another BA, the efficacy of which has been confirmed in large-scale clinical trials, is officially registered for the treatment of SLE. RTM and BLM, which have different mechanisms of action, but a common end point (B lymphocytes), occupy a key place in modern therapy for SLE. Our study presents the experience with RTM and BLM, as well as RTM and BLM combination treatment, the peculiarities of their effect on cellular immunity, and the course of the disease and points out the expected predictors of response to therapy with BAs.

Time course of changes in B-lymphocyte subpopulations during RTM therapy. The duration of the effect after a RTM treatment cycle is about 4–6 months [16]. S. Nakayamada et al. [17] note that this treatment causes a rapid depletion of naXve B cells and unswitched memory B cells, whereas PCs persist in the body for up to 28 days. The duration of remission is likely to be associated with persistent memory B cell depletion, regardless of naXve B cell repopulation. The similar results were obtained by S. Iwata et al. [18]. M.J. Leandro et al. [19] have reported that the residual cells are memory B cells and PCs after a RTX cycle. P. Roll et al. [20] have noted that there is an increase in the number of naXve B cells and a slow recovery of the memory B-cell populations after the achievement of depletion and the onset of repopulation.

Our study has yielded similar results. RTM therapy resulted in a pronounced reduction in the number of B lymphocytes mainly due to naive [17, 18] and DN memory B cells, while the residual cells were different subpopulations of memory B cells and naXve B cells, the percentage distribution of which depended on the degree B-lymphocyte depletion [19].

The number of B cells recovered more at the expense of naXve cells and memory cells [17, 18], and the proportion of switched or unswitched memory B cells prevailed at the onset of repopulation. The findings reflect the nature of cell recovery after B-lymphocyte depletion is achieved. However, B-cell subpopulations further tended to recover their baseline percentage, i.e. there was an increase in the level of naXve and DN B cells at the lower concentrations of unswitched and switched memory B cells [20]. Just the same, the long-term follow-up has shown that after successful administration of BAs there is a slow recovery of the total population of memory B cells, the number of which was significantly lower at 12 months when comparing with the baseline composition

Another task of our investigation was to search for response to RTX therapy. So, M.Y. Yusuf et al. [7] have established that

the presence of a response to BAs can be associated with the low baseline number of PCs. J.N. Anolik et al. [21] attributed these results to the degree of memory B-cell recovery. Our study has shown that partial B-lymphocyte depletion is due to the high baseline number of PCs (>0.2%), which is also confirmed by the data obtained by M.Y. Yusuf et al. [7]. These data may contribute to the better choice of therapy including RTX, the aim of which is complete cellular depletion, by either increasing the dose of the BA or using its additional infusion in patients with higher PC levels.

Time course of changes in B lymphocyte subpopulations in the therapy of BLM. Currently, there are few studies on the time course of changes in B lymphocyte subpopulations in the use of BLM. A.M. Jacobi et al. [22] have noted that BLM treatment leads to a reduction in the number of naXve, transient B cells and PCs.

The same results were obtained in the study by E. Pontarini et al. [23], in which the similar changes at 24 weeks were associated with the normalization of immunological parameters (C4, antinuclear factor, and rheumatoid factor). In turn, W. Stohl et al. [24] emphasized that BLM had no effect on memory B cells among the peculiarities of the action of the drug.

In our study, BLM therapy led to a decrease in the number of naXve B cells [22–24], and, in most patients, it reduced the total population of CD19+ B lymphocytes; however, complete B-cell depletion was not achieved. BLM treatment contributed to a decline in the proportion of PCs and PBs [22, 23], and these subpopulations were not detected in the peripheral blood of some patients at 6 months; at the same time there was a marked decrease in the immunological activity of SLE (a decrease in the level of anti-dsDNA antibodies and an increase in the content of the complement component C4 [23]. Since 3 patients were treated with BAs prior to the 9th month and followed up for 12 months; this could justify an increase in the number of PCs and PBs after the 9th month. Thus, W.G. Halpern et al. [25] demonstrated a decrease in the number of PCs in the use of BLM; however, it was reversible after its discontinuation.

Dynamics of B-lymphocyte subpopulations in RTX + BLM combination therapy. There are single studies of the efficiency of combination therapy with RTM + BLM, which mainly demonstrate the effect of BAs on clinical and laboratory parameters in patients with SLE. And only one study dealt with a detailed analysis of B-lymphocytes subpopulations in the observation of NZB/NZW F1 mice. W. Lin et al. [26] suggested that combination therapy provides a significant improvement in the course and survival in SLE due to the most effective depletion of tissue and circulating autoreactive B cells. T. Kraaij et al. [11] and F. Simonetta et al. [27] noted the high efficiency of therapy in patients with LN. R. Gualtierotti et al. [[28] reported the effectiveness of consistent use of RDM and BLM: in 3 patients, such therapy allowed to achieve long-term remission and reduce the dose of oral glucocorticoid. W. Lin et al. [26] have suggested that the combination therapy ensures a considerable improvement in the course of SLE and in survival in this disease due to the most effective depletion of tissue and circulating autoreactive B cells. Kraaij et al. [11] and F. Simonetta et al. [27] noted the high efficiency of therapy in patients with LN. R. Gualtierotti et al. [28] reported that the sequential use of RTM and BLM was effective: this therapy made it possible to achieve long-term remission and to reduce the dose of oral GCs in 3 patients.

Despite the differences in the development and severity of the disease, the combination therapy was highly effective in our patients: there was an improvement in the clinical picture, laboratory markers of SLE activity, in particular an increase in the concentration of C3 and C4 complement components and a persistent decrease in the concentration of anti-dsDNA antibodies. We first demonstrated the impact of combination therapy on a B lymphocyte subpopulation in patients with SLE. This treatment contributed to better controllability of B lymphocytes. The sequential use of BAs allowed the total population of B lymphocytes to be controlled – as a result of therapy there was their slower repopulation in patients with complete depletion after a RTX cycle and there was a continued reduction in patients with no depletion. In addition, PCs and PBs could be maintained at a low level, regardless of the primary response to RTX. At 12 months, the percentage distribution of all B lymphocyte subpopulations tended to be the baseline one. Nevertheless, when using BLM, the percentage of unswitched memory B cells increased, and naXve B cells decreased. At the same time, RTM treatment resulted in a reduction in the total population of memory B cells

REFERENCES

1. Насонов ЕЛ, Насонова ВА, редакторы. Ревматология. Национальное руководство. Москва: ГЭОТАР-Медиа; 2008. 714 с. [Nasonov EL, Nasonova VA, editors. *Revmatologiya. Natsional'noe rukovodstvo* [Rheumatology. National guidelines]. Moscow: GEOTAR-Media; 2008. 714 p.]
2. Gottschalk TA, Tsantikos E, Hibbs ML. Pathogenic inflammation and its therapeutic targeting in systemic lupus erythematosus. *Front Immunol.* 2015 Oct 28;6:550. doi: 10.3389/fimmu.2015.00550. eCollection 2015.
3. Anolik JH, Looney RJ, Lund FE, et al. Insights into the heterogeneity of human B cells: diverse functions, roles in autoimmunity, and use as therapeutic targets. *Immunol Res.* 2009 Dec;45(2-3):144-58. doi: 10.1007/s12026-009-8096-7. Epub 2009 Apr 7.
4. Александрова ЕН. В-клетки при аутоиммунных ревматических заболеваниях. В кн.: Насонов ЕЛ, редактор. Анти-В-клеточная терапия в ревматологии: фокус на ритуксимаб. Москва: ИМА-ПРЕСС; 2012. С. 8-45. [Aleksandrova EN. B-cells in autoimmune rheumatic diseases. In: Nasonov EL, editor. *Anti-V-kletochnaya terapiya v revmatologii: fokus na rituksimab* [Anti-B-cell therapy in rheumatology: focus on rituximab]. Moscow: IMA-PRESS; 2012. P. 8-45.]
5. Iwata S, Tanaka Y. B-cell subsets, signaling and their roles in secretion of autoantibodies. *Lupus.* 2016 Jul;25(8):850-6. doi: 10.1177/0961203316643172.
6. Tesar V, Hruskova Z. Belimumab in the management of systemic lupus erythematosus — an update. *Expert Opin Biol Ther.* 2017 Jul;17(7):901-908. doi: 10.1080/14712598.2017.1324846. Epub 2017 May 16.
7. Md Yusof MY, Shaw D, El-Sherbiny YM, et al. Predicting and managing primary and secondary non-response to rituximab using B-cell biomarkers in systemic lupus erythematosus. *Ann Rheum Dis.* 2017 Nov;76(11):1829-1836. doi: 10.1136/annrheumdis-2017-211191. Epub 2017 Jul 6.
8. Jacobi AM, Odendahl M, Reiter K, et al. Correlation between circulating CD27 (high) plasma cells and disease activity in patients with systemic lupus erythematosus. *Arthritis Rheum.* 2003 May;48(5):1332-42.
9. Vital EM, Dass S, Buch MH, et al. B cell biomarkers of rituximab responses in systemic lupus erythematosus. *Arthritis Rheum.* 2011 Oct;63(10):3038-47. doi: 10.1002/art.30466.
10. Jacobi AM, Reiter K, Mackay M, et al. Activated memory B cell subsets correlate with disease activity in systemic lupus erythe-

matosus delineation by expression: of CD27, IgD, and CD95. *Arthritis Rheum.* 2008 Jun; 58(6):1762-73. doi: 10.1002/art.23498.
11. Kraaij T, Huizinga TW, Rabelink TJ, Teng YK. Belimumab after rituximab as maintenance therapy in lupus nephritis. *Rheumatology (Oxford).* 2014 Nov;53(11): 2122-4. doi: 10.1093/rheumatology/keu369. Epub 2014 Sep 8.
12. Wei C, Anolik J, Cappione A, et al. A new population of cells lacking expression of CD27 represents a notable component of the B cell memory compartment in systemic lupus erythematosus. *J Immunol.* 2007 May 15;178(10):6624-33.
13. Thompson JS, Bixler SA, Qian F, et al. BAFF-R, a newly identified TNF receptor that specifically interacts with BAFF. *Science*. 2001 Sep 14;293(5537):2108-11. Epub 2001 Aug 16.
14. Liossis SC, Staveri C. B Cell-based treatments in SLE: past experience and current directions. *Curr Rheumatol Rep.* 2017 Nov 4; 19(12):78. doi: 10.1007/s11926-017-0707-z.
15. Bertsias GK, Tektonidou M, Amoura Z, et al; European League Against Rheumatism and European Renal Association-European Dialysis and Transplant Association. Joint European League Against Rheumatism and European Renal Association–European Dialysis and Transplant Association (EULAR/ERA-EDTA) recommendations for the management of adult and paediatric lupus nephritis. *Ann Rheum Dis.* 2012 Nov;71(11): 1771-82. doi: 10.1136/annrheumdis-2012-201940. Epub 2012 Jul 31.
16. Cambridge G, Isenberg DA, Edwards JC, et al. B cell depletion therapy in systemic lupus erythematosus: relationships among serum B lymphocyte stimulator levels, autoantibody profile and clinical response. *Ann Rheum Dis.* 2008 Jul;67(7):1011-6. Epub 2007 Oct 25.
17. Nakayamada S, Iwata S, Tanaka Y. Relevance of lymphocyte subsets to B cell-targeted therapy in systemic lupus erythematosus. *Int J Rheum Dis.* 2015 Feb;18(2): 208-18. doi: 10.1111/1756-185X.12534. Epub 2015 Jan 3.
18. Iwata S, Saito K, Hirata S, et al. Efficacy and safety of anti-CD20 antibody rituximab for patients with refractory systemic lupus erythematosus. *Lupus*. 2018 Apr;27(5): 802-811. doi: 10.1177/0961203317749047. Epub 2018 Jan 7.
19. Leandro MJ, Cambridge G, Ehrenstein MR, Edwards JC. Reconstitution of peripheral blood B cell after depletion with rituximab in patients with rheumatoid arthritis. *Arthritis Rheum.* 2006 Feb;54(2):613-20.
20. Roll P, Palanichamy A, Kneitz C, et al. Regeneration of B cell subsets after transient B cell depletion using anti-CD20 antibodies in rheumatoid arthritis. *Arthritis Rheum*. 2006 Aug;54(8):2377-86.
21. Anolik JH, Barnard J, Owen T, et al. Delayed memory B cell recovery in peripheral blood and lymphoid tissue in systemic lupus erythematosus after B cell depletion therapy. *Arthritis Rheum.* 2007 Sep;56(9):3044-56.
22. Jacobi AM, Huang W, Wang T, et al. Effect of long-term belimumab treatment on B cells in systemic lupus erythematosus: extension of a phase II, double-blind, placebo-controlled, dose-ranging study. *Arthritis Rheum.* 2010 Jan;62(1):201-10. doi: 10.1002/art.27189.
23. Pontarini E, Fabris M, Quartuccio L, et al. Treatment with belimumab restores B cell subsets and their expression of B cell activating factor receptor in patients with primary Sjogren's syndrome. *Rheumatology (Oxford).* 2015 Aug;54(8):1429-34. doi: 10.1093/rheumatology/kev005. Epub 2015 Mar 3.
24. Stohl W, Hiepe F, Latinis KM, et al. Belimumab reduces autoantibodies, normalizes low complement, and reduces select B-cell populations in patients with systemic lupus erythematosus. *Arthritis Rheum.* 2012 Jul; 64(7):2328-37. doi: 10.1002/art.34400.
25. Halpern WG, Lappin P, Zanardi T, et al. Chronic administration of belimumab, a BLyS antagonist, decreases tissue and peripheral blood B-lymphocyte populations in cynomolgus monkeys: pharmacokinetic, pharmacodynamics and toxicologic effects. *Toxicol Sci.* 2006 Jun;91(2):586-99. Epub 2006 Mar 3.
26. Lin W, Seshasayee D, Lee WP, et al. Dual B cell immunotherapy is superior to individual anti-CD20 depletion or BAFF blockade in murine models of spontaneous or accelerated lupus. *Arthritis Rheumatol.* 2015 Jan; 67(1):215-24. doi: 10.1002/art.38907.
27. Simonetta F, Allali D, Roux-Lombard P, Chizzolini C. Successful treatment of refractory lupus nephritis by the sequential use of rituximab and belimumab. *Joint Bone Spine.* 2017 Mar;84(2):235-236. doi: 10.1016/j.jbspin.2016.01.008. Epub 2016 May 26.
28. Gualtierotti R, Borghi MO, Gerosa M, et al. Successful sequential therapy with rituximab and belimumab in patients with active systemic lupus erythematosus: a case series. *Clin Exp Rheumatol.* 2018 Jul-Aug;36(4): 643-647. Epub 2018 Feb 27.

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