Predicting the Efficacy of Anti-B-Cell Therapy in Patients with Multiple Sclerosis

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Abstract

Introduction. *Prognostic markers can be used to evaluate a response to anti-B-cell therapy in patients with multiple sclerosis (MS).*

Aim. *The study aimed to evaluate the characteristics of peripheral blood (PB) lymphocytes and monocytes in patients with aggressive MS during the first 6 months of anti-B-cell therapy.*

Materials and methods. *Twenty-nine patients with aggressive MS were treated with a humanized anti-CD20 monoclonal antibody (anti-CD20 mAb). A panel of MAbs to differentiation antigens of PB lymphocytes was used to assess the parameters of cellular immunity using six-color flow cytometry. The reference values were based on the similar parameters of ten apparently healthy volunteers.*

Results. *At month 6, the initial course of anti-CD20 therapy resulted in low recovery of the PB sub-populations of B cells in 85% of patients. Significant decreases were reported in the absolute counts of T cells, T helper cells, Natural Killer (NK) cells, and relative percentage of natural killer T (NKT) cells. The study also showed low levels of activated T cells and significantly decreased percentage of memory B cells ($CD27^+$) and B cells expressing costimulatory and activation molecules ($CD40^+$, $CD38^+$, and $CD25^+$, respectively). A significant decrease in the mean fluorescence intensity of HLA-DR was observed on PB monocytes compared to normal values and those in patients receiving other disease-modifying therapies. Anti-CD20 therapy may indirectly suppress their antigen-presenting ability. Other immunological criteria for prediction of MS progression and magnetic resonance imaging activity during the first year of anti-B-cell therapy may include the following changes from baseline: increased percentages of $CD3^+$, $CD3^+HLA\text{-}DR^+$, $CD25^+CD3^+$, and $CD95^+CD3^+$ cells; significant expression of the CD40 molecule and B-cell activation markers CD38 and CD25, and decreased expression of CD95.*

Conclusion. *Further research on changes in cellular immunity parameters during anti-CD20 therapy could allow for early adjustment of MS treatment to stabilize the patient's condition.*

Keywords: *multiple sclerosis; T lymphocytes; B lymphocytes; biomarkers*

Ethics approval. The study was approved by the Local Ethics Committee at M.F. Vladimirsky Moscow Region Research Clinical Institute (Protocol No. 8 dated June 13, 2019).

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Прогнозирование эффективности лечения пациентов с рассеянным склерозом, получающих анти-В-клеточную терапию

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Аннотация

Введение. *При назначении анти-В-клеточной терапии больным рассеянным склерозом (РС) оценить ответ на терапию можно по уровню прогностических маркеров.*

Цель: *исследование особенностей популяций лимфоцитов и моноцитов периферической крови (ПК) у пациентов с агрессивным РС в первые 6 мес проведения анти-В-клеточной терапии.*

Материалы и методы. *29 пациентам с агрессивным РС было назначено гуманизированное анти-CD20 моноклональное антитело (анти-CD20-МАТ). Параметры клеточного иммунитета оценивали методом 6-цветной проточной цитометрии с использованием панели МАТ к дифференцировочным антигенам лимфоцитов ПК. В качестве референсных значений использованы аналогичные показатели 10 практически здоровых лиц.*

Результаты. *Проведение 1-го курса анти-CD20-МАТ продемонстрировало низкую степень восстановления количественных параметров популяции В-лимфоцитов ПК у 85% пациентов через 6 мес. Было отмечено выраженное снижение абсолютного количества Т-лимфоцитов, Т-хелперной субпопуляции, NK-лимфоцитов, содержания NKT-субпопуляции и низкий уровень активированных Т-лимфоцитов, существенное снижение содержания В-клеток-памяти ($CD27^+$), а также В-клеток, экспрессирующих костимулирующие и активационные молекулы ($CD40^+$, $CD38^+$, $CD25^+$ соответственно). Обнаружено значительное снижение параметра средней интенсивности флюоресценции HLA-DR на моноцитах ПК по сравнению с нормальными значениями и пациентами, получавшими другие препараты, изменяющие течение РС. Возможно, анти-CD20-МАТ опосредованно подавляет их антигенпрезентирующую способность. Иммунологическими дополнительными критериями прогнозирования обострения РС и активности по МРТ в первый год анти-В-клеточной терапии могут служить изменения по сравнению с исходными следующих параметров: повышение содержания $CD3^+$-, $CD3^+HLA\text{-}DR^+$-, $CD25^+CD3^+$-, $CD95^+CD3^+$-лимфоцитов; выраженная экспрессия костимулирующей молекулы CD40 и маркеров активации В-лимфоцитов CD38, CD25 при снижении экспрессии CD95.*

Заключение. *Дальнейшее изучение динамики изменений показателей клеточного иммунитета под действием анти-CD20-МАТ даст возможность ранней коррекции терапии РС, направленной на стабилизацию состояния пациента.*

Ключевые слова: *рассеянный склероз; Т-лимфоциты; В-лимфоциты; биомаркеры*

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Introduction

Multiple sclerosis (MS) is considered a heterogeneous, multifactorial, immune-mediated disease, with T cells and B cells both playing a key role in its pathogenesis. B cells present antigens to T cells and produce cytokines, which act as inflammatory mediators [1, 2]. B cells also produce autoimmune antibodies to myelin components, thereby affecting the processes of demyelination and axonal damage. In other words, B cells are involved in the humoral immune response at every stage of MS [3].

Treatment strategies for MS are based on its pathogenesis and include reducing the activity of Th1/Th17 cells, activating regulatory T cells, inhibiting the migration of lymphocytes into the nervous system, and targeting

B cells. The mechanisms of autoimmune inflammation, including the role of regulatory B cells, are not fully understood [4, 5].

The research focuses on anti-B-cell therapies, particularly the use of anti-CD20 monoclonal antibodies (mAbs). This protein is expressed on pre-B cells, mature B cells, and memory B cells, but not on early B cell progenitors or plasma cells. Anti-CD20 mAbs deplete B cells via antibody-dependent mechanisms such as phagocytosis, cellular cytotoxicity and apoptosis, resulting in reduced immunopathological inflammation in MS. However, humoral and innate immunity, as well as the ability to recover B cells, are still maintained, and the total T cell count remains almost unchanged [6–9].

Experimental and clinical studies focus on mechanisms by which anti-CD20 therapy depletes and recovers B-cell-rich immunological compartments, such as the bone marrow, peripheral blood (PB), spleen, and lymph nodes, as well as to evaluate B cell function after temporary elimination [10, 11]. The most effective B cell depletion was observed in blood, where anti-CD20 therapy resulted in virtually complete absence of B cells. The lowest depletion of $CD19^+$ B cells was reported in the bone marrow. After treatment, $CD20^+$ B cells repopulated the bone marrow and spleen simultaneously, and then reappeared in the blood [10].

One of the key issues related to prescribing anti-B-cell therapy is to establish personalized criteria, such as prognostic markers for optimal treatment response, as well as treatment dosage and frequency. For some patients, frequency of every six months may be excessive due to a significant decrease the $CD19^+$ B cell count. Therefore, the percentage of memory B cells ($CD19^+CD27^+$) was considered such a prognostic marker [12–14].

In real-world practice, it is particularly relevant to evaluate the effects of anti-CD20 therapy on various parameters of innate and adaptive cellular immunity in patients with MS, especially PB parameters, to assess the systemic effects of therapy.

The study **aimed** to evaluate the characteristics of PB lymphocytes and monocytes in patients with aggressive MS during the first 6 months of anti-B-cell therapy.

Materials and methods

Inclusion criteria:
- informed consent signed by a patient;
- aggressive course of MS;
- anti-CD20 therapy for at least 24 months;
- availability of testing for cellular immunity before the start of therapy and 6 months after the first dose.

Exclusion criteria:
- refusal to sign an informed consent;
- contraindications to anti-CD20 therapy;
- inability to attend follow-up visits.

A control group included 10 apparently healthy volunteers who were matched by sex and age.

The study was conducted in accordance with the Helsinki Declaration of the World Medical Association, the International Council for Harmonization, and local laws. All study participants provided informed written consent after a detailed discussion of the protocol. The study was approved by the Local Ethics Committee at M.F. Vladimirsky Moscow Region Research Clinical Institute (Protocol No. 8 dated June 13, 2019).

Patients with MS (n = 29) were examined according to clinical guidelines[1] to identify contraindications to anti-B-cell therapy with a recombinant humanized mAb that selectively targets B cells expressing CD20 (anti-CD20 mAbs). The first dose of anti-CD20 therapy was administered via a specialized system as two intravenous 300-mg infusions, the second of which was given two weeks after the first dose. This was followed by single 600-mg infusions every six months. PB parameters of cellular immunity parameters were evaluated before the initial course of anti-CD20 therapy and during subsequent courses at 6, 12, 18, and 24 months thereafter, with changes in functional state and neuroimaging assessed.

Six-color flow cytometry was performed to evaluate the cellular immune response in patients with MS, using a panel of mAbs (Becton Dickinson) to differentiate antigens on PB lymphocytes and monocytes.

The percentages and counts of the following cell types were assessed: T-cells ($CD3^+$), B-cells ($CD19^+CD20^+$), Natural Killer (NK) cells ($CD3^-CD16^+CD56^+$), T helper cells ($CD3^+CD4^+$), cytotoxic T cells ($CD3^+CD8^+$), and Natural Killer T (NKT) cells ($CD3^+CD16^+CD56^+$), as well as the percentages of activated T-cells ($CD3^+CD25^+$, $CD3^+$HLA-DR^+, $CD3^+CD95^+$) within the $CD45^+$ lymphocyte population. HLA-DR expression on PB monocytes was evaluated based on the $CD14^+$HLA-DR^+ count. HLA-DR expression intensity on PB monocytes was evaluated using mean fluorescence intensity (MFI), which reflects HLA-DR molecule density on the monocyte membrane and indicates their antigen-presenting function.

The study evaluated the immunophenotypic characteristics and timing of B cell recovery in PB after anti-CD20 therapy. Based on cytometry of the entire lymphocyte population using the $CD45^+$/side scatter gating with no more than 50,000 events to be included, it was assumed that not all patients with MS would have detectable B cells after six months of the initial course of anti-CD20 therapy. Indeed, the majority of patients (85%) had no $CD19^+$ cells. The cell elements were concentrated to collect a large number of B cells, forming a complete cluster. The PB samples were preliminarily divided into several portions of 500–1,000 μL each. The erythrocytes were lysed using PharmLyse solution (Becton Dickinson). After rinsing twice with phosphate-buffered saline, the sediment was concentrated in one test tube. The next step was to perform cytometry on the PB samples that had been incubated with mAbs to specific lymphocyte differentiation antigens (CDs), conjugated with fluorescent dyes. B cells were gated based on side scatter (SSC) and $CD19^+$ expression. The analysis included at least 500,000 events.

[1]Multiple sclerosis. Clinical guidelines, 2025. https://cr.minzdrav.gov.ru/clin-rec (accessed on August 1, 2025).

As a result, the composition of the B cell subpopulations was determined within the $CD19^+$ lymphocyte gate: B1 cells ($CD5^+$), memory B cells ($CD27^+$), and expression of activation and costimulatory molecules (CD40, CD25, CD38, and CD95).

Statistical analysis was performed using SPSS Statistics v. 23 (IBM Corp.). The Kolmogorov–Smirnov test was used to evaluate the data normality. The following tests were used to identify differences between groups: the independent t-test, the Mann–Whitney test, the one-sample t-test with one-way analysis of variance (ANOVA), and the paired t-test with the Wilcoxon test (differences were considered significant at $p < 0.05$). A regression analysis was performed that included the Durbin–Watson pretest and partial correlations to evaluate the strength of dependence between the variables (the effects were considered significant at $p < 0.05$). Discriminant analysis was used to identify the most significant difference factor for nominal variables in independent samples. The analysis included Wilks lambda, one-way ANOVA, the F-test for equality of variances, and stepwise regression. The quantitative data on the HLA-DR expression on PB monocytes are presented as the median and quartiles (Me [Q_1; Q_3]). A Kruskal–Wallis test was used for comparison. Differences between samples with a less than 5% probability of a type I error ($p < 0.05$) were considered significant. In cases of multiple pairwise comparisons, the Bonferroni correction was used.

Results

MS therapy was adjusted with anti-CD20 mAbs in 29 patients in the study group, including 22 women (75.9%) and 7 men (24.1%). The mean age was 36.9 ± 7.7 years. The mean duration of MS was 15.7 years (95% confidence interval [CI]: 6.42, 24.91). The mean Expanded Disability Status Scale (EDSS) score was 3.2 ± 1.5. Since their MS diagnosis, patients had received an average of 3.7 disease-modifying therapies (DMTs) (95% CI: 2.83, 4.59).

Of 29 patients with aggressive MS included in the study, six (23.6%) received interferon beta, 19 (65.2%) received natalizumab, one (3.4%) received teriflunomide, one (3.4%) received glatiramer acetate, and two (6.9%) treatment-naïve patients initiated anti-CD20 therapy.

Of 19 patients who received natalizumab as their last treatment option before treatment adjustment, 14 switched due to a high antibody titer to the John Cunningham virus for more than 24 months of treatment, and high risk for progressive multifocal leukoencephalopathy. Four patients reported a worsening of MS symptoms and progressed to secondary progressive MS. A washout period for natalizumab switch to an anti-CD20 mAb was 7.4 months (95% CI: 4.14, 10.71). Twenty (71.3%) patients included in the study group had experienced a MS exacerbation within the previous 12 months, including patients who were in the washout period after natalizumab discontinuation.

The total PB lymphocyte count before anti-CD20 therapy was significantly higher ($p = 0.001$) than that of healthy volunteers (Table 1). This difference may be due to the previous use of natalizumab, which could be associated with increased PB lymphocyte counts [15].

Compared to normal values, the study group showed a significant increase in the percentage of T helper cells, though not in their absolute counts. There was also a decrease in the percentage of cytotoxic T cells and NKT cells, as well as in absolute counts of NK cells. Levels of T cells and B cells, as determined by CD19 and CD20 markers, as well as their antigen-presenting potential ($CD19^+HLA\text{-}DR^+$), were normal. However, patients demonstrated a significant decrease in activated T cells ($CD3^+HLA\text{-}DR^+$) before the adjustment of anti-CD20 therapy.

Six months after the initial course of anti-CD20 therapy, the total count of PB lymphocytes decreased significantly compared with baseline values and those of the control group (Table 1). However, this decrease did not reach the values determined by the Common Toxicity Criteria (CTC) score.[2] As for the relative parameters, patients showed a significant increase in PB levels of T cells and T helper cells. However, this increase was associated with compensatory mechanisms due to the depletion of B cells, which had not normalized by the start of the study. These parameters showed significantly lower absolute values compared to the control group. The level of the cytotoxic T cells did not differ significantly from that of healthy volunteers, in either relative or absolute terms. There was a significant decrease in the absolute count of NK cells and percentage of NKT cells, as well as low levels of activated T cells. NK cells are considered to play a double role in MS. First, they can demonstrate a cytotoxic effect on autoreactive effector cells. Second, they can promote the damage and lysis of astrocytes and oligodendrocytes, as well as influence the level of regulatory T cells in patients with MS [16].

The percentage of B cells in patients ranged from 0% to 7.5%. Only 15% of patients had normal values within the range of 6.0% to 7.5%. Thirty-eight percent of patients had B cell percentages of 2% to 4%. The remaining 47% had a percentage of up to 1%. Therefore, 85% did not normalize their B cell levels. Similarly, the percentage of B cells that could present antigens ($CD19^+HLA\text{-}DR^+$) decreased dramatically.

Before the start of anti-CD20 therapy, patients with MS did not differ significantly from the control group in terms of the percentage of B cells expressing the costimulatory molecule CD40, the CD95 apoptosis marker, or the B1 cell subpopulation ($CD19^+CD5^+$) associated with the production of autoantibodies (Table 2).

Patients with MS showed a significant ($p < 0,05$) increase in the percentage of memory B cells ($CD19^+CD27^+$) and B cells activated by CD38 and CD25 antigens compared to the control group.

At month 6 after the initial course of anti-CD20 therapy, patients with MS showed a significant increase in the percentage of circulating $CD5^+$ B cells ($p < 0.05$) and a significant decrease in the percentage of memory B cells ($CD27^+$), as well as B cells expressing costimulatory and activation molecules (CD40, CD38, and CD25) ($p < 0.05$).

[2]Multiple sclerosis. Clinical guidelines, 2025. URL: https://cr.minzdrav.gov.ru/clin-rec

Table 1. Cellular immunity parameters in patients with MS ($M \pm SD$)

Parameter		Before B-cell therapy (n = 29)	Six months after the first dose of anti-CD20 therapy (n = 29)	Healthy volunteers (n = 10)	p
		1	2	3	
Total lymphocyte count	× 10^9/L	2,145.6 ± 784.3	1,725.4 ± 648.9	2,070.0 ± 101.3	$p_{1-2} < 0.001$* $p_{1-3} = 0.001$* $p_{2-3} = 0.001$*
$CD3^+$ T cells	%	75.67 ± 6.71	85.03 ± 5.63	74.97 ± 1.53	$p_{1-2} = 0.001$* $p_{1-3} = 0.09$ $p_{2-3} = 0.001$*
	abs.	1520.7 ± 118.2	1,352.9 ± 65.9	1,670.7 ± 131.5	$p_{1-2} = 0.107$ $p_{1-3} = 0.1230$ $p_{2-3} = 0.0173$
T helper cells $CD3^+CD4^+$	%	46.84 ± 7.75	51.54 ± 10.64	40.69 ± 2.27	$p_{1-2} = 0.001$* $p_{1-3} < 0.001$* $p_{2-3} = 0.001$*
	abs.	939.2 ± 71.9	795.8 ± 64.4	1,125.5 ± 103.2	$p_{1-2} = 0.0678$ $p_{1-3} = 0.6870$ $p_{2-3} = 0.0044$*
Cytotoxic T cells $CD3^+CD8^+$, % of cells	%	26.89 ± 7.96	31.43 ± 10.51	33.00 ± 3.08	$p_{1-2} = 0.05$ $p_{1-3} < 0.001$* $p_{2-3} = 0.484$
	abs.	565.7 ± 75.6	477.6 ± 49.7	537.9 ± 56.4	$p_{1-2} = 0.1633$ $p_{1-3} = 0.3820$ $p_{2-3} = 0.2076$
NK cells $CD3^-CD16^+$ $CD56^+$	%	11.50 ± 4.46	12.76 ± 6.09	12.47 ± 1.70	$p_{1-2} = 0.001$* $p_{1-3} = 0.281$ $p_{2-3} = 0.821$
	abs.	189.0 ± 25.2	180.3 ± 17.2	328.7 ± 18.4	$p_{1-2} = 0.3857$ $p_{1-3} < 0.0000$* $p_{2-3} < 0.0000$*
NKT cells $CD3^+CD16^+CD56^+$	%	6.01 ± 4.52	5.7 ± 3.68	10.50 ± 1.73	$p_{1-2} = 0.001$* $p_{1-3} = 0.001$* $p_{2-3} = 0.001$*
B cells $CD19^+$	%	12.78 ± 4.8	1.60 ± 2.25	11.79 ± 1.86	$p_{1-2} = 0.001$* $p_{1-3} = 0.290$ $p_{2-3} < 0.001$*
	abs.	284.7 ± 60.3	25.7 ± 10.0	271.6 ± 29.6	$p_{1-2} < 0.001$* $p_{1-3} = 0.4214$ $p_{2-3} < 0.001$*
B cells $CD20^+$	%	12.25 ± 3.44	0.75 ± 1.37	11.19 ± 1.83	$p_{1-2} < 0.001$* $p_{1-3} = 0.723$ $p_{2-3} < 0.001$*
$CD3^+HLA\text{-}DR^+$	%	7.67 ± 3.08	6.11 ± 2.52	13.30 ± 2.60	$p_{1-2} = 0.001$* $p_{1-3} = 0.001$* $p_{2-3} < 0.001$*
$CD19^+HLA\text{-}DR^+$	%	9.86 ± 5.3	1.30 ± 1.94	10.32 ± 3.41	$p_{1-2} = 0.001$* $p_{1-3} = 0.709$ $p_{2-3} < 0.001$*
$CD3^+CD25^+$	%	19.6 ± 6.64	26.81 ± 23.83	11.16 ± 5.22	$p_{1-2} = 0.06$ $p_{1-3} = 0.056$ $p_{2-3} = 0.35$
$CD3^+CD95^+$	%	10.5 ± 6.85	44.42 ± 25.05	42.14 ± 18.99	$p_{1-2} < 0.001$* $p_{1-3} = 0.69$ $p_{2-3} = 0.96$

Note. The results are presented as the relative percentage (%) and absolute (abs.) count of lymphocytes. The relative value is expressed as a percentage of the total count of $CD45^+$ cells, and the absolute value is expressed as the number of cells in 1 μL of PB. *The difference is statistically significant when adjusted by the Bonferroni correction.

Table 2. Parameters of B cell immunity in patients with MS (% of $CD19^+$ gate cells)

Lymphocyte population	Before B-cell therapy (n = 29)	Six months after the first dose of anti-CD20 therapy (n = 29)	Healthy volunteers (n = 10)	p
	1	2	3	
$CD40^+$	48.32 ± 27.49	17.13 ± 23.12	49.20 ± 4.45	p_{1-2} = 0.001* p_{1-3} = 0.883 p_{2-3} = 0.001*
$CD95^+$	23.02 ± 17.32	25.85 ± 25.05	19.89 ± 2.30	p_{1-2} = 0.004* p_{1-3} = 0.406 p_{2-3} = 0.427
$CD5^+$	14.35 ± 11.10	21.38 ± 20.21	17.29 ± 3.96	p_{1-2} = 0.004* p_{1-3} = 0.228 p_{2-3} = 0.497
$CD27^+$	37.40 ± 15.32 ↑	25.45 ± 18.15	28.30 ± 2.74	p_{1-2} = 0.001* p_{1-3} = 0.01* p_{2-3} = 0.599
$CD38^+$	34.41 ± 20.8 ↑	29.04 ± 23.24 ↑	16,10 ± 2.63	p_{1-2} = 0.001* p_{1-3} = 0.001* p_{2-3} = 0.08
$CD25^+$	23.16 ± 14.59	13.93 ± 12.83	13.93 ± 12.82	p_{1-2} = 0.003* p_{1-3} = 0.007* p_{2-3} = 0.970

Note. *The difference is statistically significant after the Bonferroni correction.

A significant increase in the expression of CD95 and CD5 antigens on B cells from baseline levels ($p < 0.05$) suggests an inadequate response to a single dose of anti-CD20 therapy and high likelihood of a future MS exacerbation.

The study evaluated how different DMTs affect the expression patterns of a HLA-DR molecule on PB monocytes in patients with MS. No significant differences in the percentages of monocytes expressing HLA-DR were found between treatment-naïve patients with aggressive MS, all DMT groups, and healthy volunteers (Table 3). The MFI HLA-DR index in treatment-naïve patients and patients treated with interferon-beta and natalizumab did not differ from that of healthy volunteers. A significant decrease in MFI HLA-DR was only found in patients who received the initial course of anti-CD20 therapy compared to healthy volunteers. This finding suggests that anti-CD20 therapy may indirectly affect PB monocytes by suppressing their ability to present antigens.

The study group (n = 29 patients) continued to be followed up for 24 months, receiving subsequent courses of anti-CD20 therapy at the dose of 600 mg at 6, 12, 18, and 24 months after the initial course.

MRI activity was recorded in 7 patients (24.13%). In 1 patient, gadolinium-enhancing (Gd^+) lesions were detected after 6 and 12 months of therapy. Changes in MRI scans revealed Gd^+ lesions in 2 patients after 6 months, 3 patients after 12 months, and 1 patient after 24 months of therapy. New non-contrast-enhancing lesions were detected in two patients: one after 12 months and the other after 24 months.

Clinical data, disease progression, and cellular immunity parameters in patients before and six months after the start of anti-CD20 therapy revealed changes in PB lymphocyte subpopulations, which can be interpreted as risk factors for MS activity within two years. An increase in the relative percentage of $CD3^+$ T cells in a follow-up test is a significant factor for MRI activity during the first six months of therapy (p = 0.014, predictive accuracy of 68.2%). A decrease in $CD19^+$ and $CD20^+$ counts was not identified as significant factors (p = 0.09).

At month 12, MRI activity with prognostic accuracy of greater than 90% was detected in patients who showed increased levels of $CD25^+CD3^+$ (p = 0.007), $CD95^+CD3^+$ (p = 0.046), $CD40^+CD19^+$ (p = 0.000), $CD5^+CD19^+$ (p = 0.003), and $CD38^+CD19^+$ (p = 0.001) cells, as well as decreased levels of $CD95^+CD19^+$ cells (p = 0.001) after the first course of anti-CD20 therapy. This study revealed no significant biomarkers for MRI activity at month 24, which may be due to PB changes resulting from subsequent courses of anti-B-cell therapy.

An exacerbation was diagnosed in five (17.24%) patients and in one (3.44%) patient after 6 and 12 months of therapy. Increased levels of activated $CD3^+HLA\text{-}DR^+$ cells was a significant risk factor for exacerbations after 6 months of therapy, with a 90.9% probability (p = 0.02).

During 12 months after the start of therapy, the risk of exacerbations increased by more than 90% with increased levels of $CD25^+CD3^+$ (p = 0.01), $CD95^+CD3^+$ (p = 0.05), $CD40^+CD19^+$ (p = 0.000), $CD5^+CD19^+$ (p = 0.003), and

Table 3. HLA-DR expression on PB monocytes, Me [Q_1; Q_3]

Parameter	Healthy volunteers (*n* = 10)	Treatment-naïve patients with aggressive MS (*n* = 2)	Patients receiving interferon beta (*n* = 6)	Patients receiving natalizumab (*n* = 19)	Patients receiving the initial course of anti-CD20 therapy (*n* = 29)	*p*
	1	2	3	4	5	
Monocytes CD14⁺ HLA-DR⁺, % of cells	95.4 [93.1; 100.0]	96.1 [89.7; 97.1]	94.8 [90.4; 96.7]	92.4 [86.9; 96.0]	91.0 [81.4; 94.6]	$p_{1–2}$ = 0.152 $p_{1–3}$ = 0.101 $p_{1–4}$ = 0.123 $p_{1–5}$ = 0.082
MFI HLA-DR	230.4 [198.0; 261.7]	186.1 [115.0; 256.3]	207.3 [83.8; 263.3]	160.2 [126.1; 227.6]	111.6 [100.4; 139.5]	$p_{1–2}$ = 0.148 $p_{1–3}$ = 0.179 $p_{1–4}$ = 0.137 $p_{1–5}$ = 0.008*

Note. *The difference is statistically significant after the Bonferroni correction.

CD38⁺CD19⁺ (p = 0.001) cells. In addition, decreased levels of CD95⁺CD19⁺ cells (p = 0.001) were reported (see Figure). This study did not identify any significant biomarkers of potential exacerbation after 24 months.

Four patients (13.7%) demonstrated MS progression: three (10.3%) during the first year of therapy and one (3.4%) during the second year. No significant changes in immune status parameters were identified as factors for MS progression after the first course of anti-CD20 therapy.

Discussion

Numerous experimental and clinical studies of cellular immunity in patients with MS have shown high variability in all study lymphocyte subpopulations, particularly during DMT compared to untreated patients [14, 15, 18]. The proinflammatory and encephalitogenic effects of CD8⁺ T cells increased upon contact with myelin basic protein molecules [16]. In addition, the percentages of T cells (CD3⁺) and T helper cells (CD4⁺), including activated ones (CD4⁺HLA-DR⁺), decreased, while the percentage of NKT cells (CD3⁺CD16⁺CD56⁺) increased. The increased count of activated CD8⁺ cytotoxic T cells suggests their crucial role in MS progression.

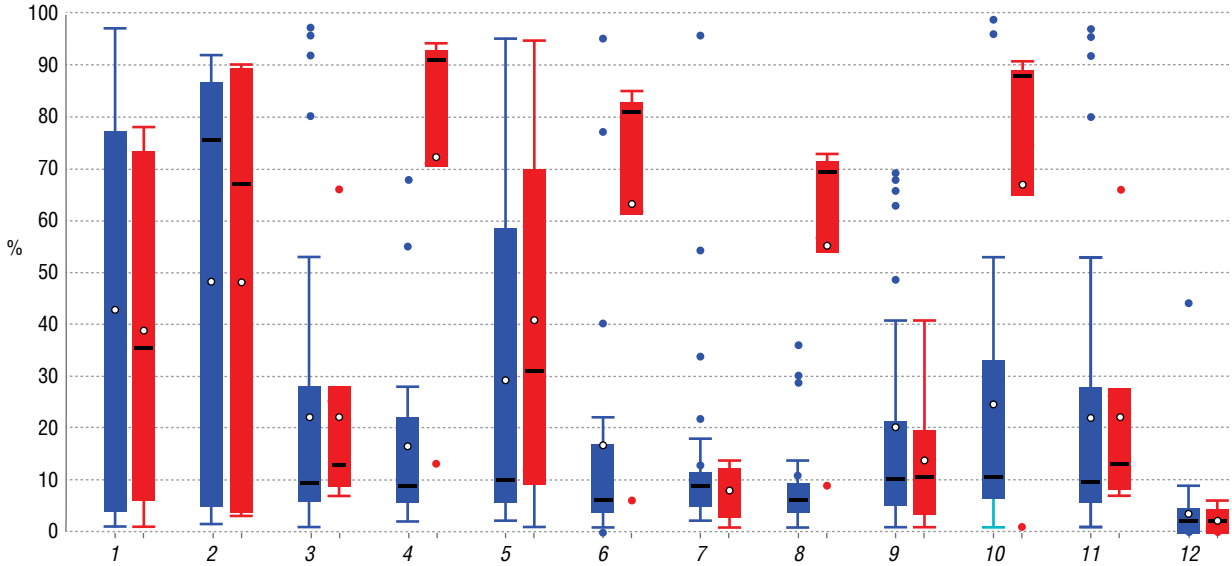
Our study revealed significant changes in baseline cellular immunity parameters in patients with MS compared to healthy volunteers. These changes included the increased percentage of T helper cells (with no change in the absolute count), decreased percentage of cytotoxic T cells and NKT cells, and decreased count of NK cells and activated (CD3⁺HLA-DR⁺) T cells. These patients showed a significant increase in the percentage of memory B cells (CD19⁺CD27⁺), as well as B cells activated by CD38 and CD25 antigens. These changes were correlated with the clinical presentation: at the time of inclusion, 20 (71.3%) patients had experienced an MS exacerbation within the previous 12 months. All examined patients with MS required therapy adjustment.

Patients who received the first course of anti-CD20 therapy showed extremely heterogeneous levels of recovery of quantitative B-cell parameters. By the time of the second dose of anti-CD20 therapy at month 6, 85% of patients had not normalized their B cell counts.

Six months after the first course of anti-B-cell therapy, patients showed a more than tenfold decrease in absolute and relative B-cell parameters, as well as a decrease in total PB lymphocyte counts due to a decrease in the absolute T-cell and T-helper counts, although their percentages increased. In addition, the study reported a significant decrease in the absolute count of NK cells and the percentage of NKT cells, an even lower level of activated T cells (CD3⁺HLA-DR⁺), and a significant decrease in memory B cells (CD27⁺), including the main pool of B cells expressing costimulatory and activation molecules (CD40, CD38, CD25). Therefore, patients with MS demonstrated a significant decrease in the absolute PB counts of key immunocompetent cells, including T cells and NK cells, even after the first course of anti-CD20 therapy. There was also inhibition of the activation potential of T and B cells, including their ability to present antigens.

The interaction between the costimulatory molecule CD40 on B cells and its ligand, CD40L (CD154), on T cells stimulates an immune response and activates B cells to produce antibodies [18]. The presence of CD40 on B cells within inflammatory lesions of the central nervous system in deceased patients with MS suggests that antibody production through interactions between T cells and B cells via CD40 may contribute to the development of the condition [19]. A decrease in CD40⁺ B cells during anti-CD20 therapy may indicate the reduced ability of these cells to trigger an immune response. Therefore, it can be concluded that the anti-CD20 therapy affects mature CD20⁺ B cells directly and indirectly by affecting the costimulatory molecule on these cells and the T-cell component.

In addition, in this group of patients, the expression patterns of HLA-DR on PB monocytes suggest a significant decrease in the density of this molecule, as determined by MFI. This may indicate suppression of the antigen-presenting ability of the monocyte/macrophage component of the immune system. In neuroinflammation and central nervous system lesions in MS,



Parameters of immune status in patients with MS.
1: $CD25^+CD3^+$ at baseline; *2*: $CD25^+CD3^+$ at month 6; *3*: $CD95^+CD3$ baseline; *4*: $CD95^+CD3$ at month 6; *5*: $CD40^+CD19$ at baseline; *6*: $CD40^+CD19$ at month 6; *7*: $CD5^+CD19^+$ at baseline; *8*: $CD5^+CD19^+$ at month 6; *9*: $CD38^+CD19$ at baseline; *10*: $CD38^+CD19$ at month 6; *11*: $CD95^+CD19^+$ at baseline; *12*: $CD95^+CD19^+$ at 6 months. Blue bars: patients with no exacerbation. Red bars: patients who experienced an exacerbation after the first dose of therapy.

macrophages and microglia can act as antigen-presenting cells, promoting the activation of effector lymphocytes and stimulating a specific immune response [20]. Macrophage involvement in the pathogenesis of MS may also influence disease progression. PB monocytes are innate immune cells that differentiate into tissue macrophages when they migrate into tissues and act as specialized antigen-presenting cells because they express class II major histocompatibility complex antigens, which increases when the cells are activated. Antigen presentation is a critical component of the immune response, that links the innate and adaptive immune systems [21]. Therefore, research on the antigen-presenting ability of monocytes can significantly improve our understanding of the MS immunopathogenesis.

Clinical and experimental data suggest that monocytes with low or absent HLA-DR expression cannot properly present antigens and produce inflammatory mediators in response to appropriate stimuli [23].

Some publications report that a decrease in HLA-DR expression on monocytes is associated with a high risk of infectious complications in patients with trauma, burns, pancreatitis, or sepsis, as well as in patients undergoing organ transplantation or heart and vascular surgery [24–28].

Statistical methods were established to determine the association between changes in quantitative parameters of T and B cell subpopulations and significant risk factors for an unfavorable long-term MS prognosis. These parameters included the increased percentage of $CD3^+$ cells, subpopulations of $CD3^+HLA\text{-}DR^+$, $CD25^+CD3^+$, $CD95^+CD3^+$, $CD40^+CD19^+$, $CD5^+CD19^+$, and $CD38^+CD19^+$ cells, as well decreased levels of $CD95^+CD19^+$ cells. Therefore, increased activation of T cells and B cells, as well as decreased B cell apoptosis, are significant risk factors for MS exacerbation and MRI activity in the future.

Conclusion

At month 6, the initial course of anti-CD20 therapy resulted in low recovery of the B cell sub-populations in 85% of patients. A relative increase in the percentage of total T cells was revealed as a result of B cell depletion. In addition, significant decreases were observed in the absolute values of primary parameters of T cells and NK cells, as well as counts of activated T cells and B cells. These results may indicate an insufficient ability of recovering B cells to present antigens and provide the complete immune response.

The ability of monocytes and macrophages to present antigens was suppressed after the first dose of anti-CD20 therapy, but in our study, this effect was not observed in treatment-naïve patients with aggressive MS or during other types of DMTs. These facts may indicate the high efficacy of the first course of anti-CD20 therapy. Further analysis is required to correlate these facts with the clinical characteristics of patients in this group.

Additional immunological criteria for predicting MS exacerbation and MRI activity in the first year of therapy may include changes from baseline in the following immunological parameters: increased percentages of $CD3^+$ cells and subpopulations of $CD3^+HLA\text{-}DR^+$, $CD25^+CD3^+$, $CD95^+CD3^+$, $CD40^+CD19^+$, $CD5^+CD19^+$, and $CD38^+CD19^+$ cells; decreased levels of $CD95^+CD19^+$ cells.

Further research on biomarkers allows for the early adjustment of MS therapy, thereby stabilizing the patient's condition.

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