ORIGINAL INVESTIGATIONS

T- and B-lymphocytes subpopulations in the early and advanced rheumatoid arthritis

Martynova A.V.[1], Popkova T.V.[1], Aleksankin A.P.[2], Gridneva G.I.[1], Gerasimova E.V.[1], Gorbunova Yu.N.[1], Semashko A.S.[1], Lila A.M.[1,3]

[1]V.A. Nasonova Research Institute of Rheumatology, Moscow;
[2]Research Institute of Human Morphology, Moscow;
[3]Department of rheumatology Russian Medical Academy of Continuing Professional Education, Moscow
[1]34A, Kashirskoe Shosse, Moscow 115522, Russia; [2]3, Tsyurupy, Moscow 117418, Russia;
[3]2/1, Barrikadnaya St., Build. 1, Moscow 125993, Russia

Objective: *to evaluate changes in T- and B-lymphocyte subpopulations at different stages of rheumatoid arthritis (RA).*
Patients and methods. *The study included 53 patients with a definite RA diagnosis according to the 2010 ACR/EULAR criteria (mean age 54.2 [47; 62] years). Group 1 included 27 patients (25 women and 2 men) without history of synthetic disease modifying anti-rheumatic drugs (sDMARDs) intake, group 2 included 26 patients (22 women and 4 men) receiving sDMARDs (methotrexate or leflunomide). The control group consisted of 29 healthy volunteers (23 women and 6 men), the median age was 58.5 [53; 62] years. In all participants flow cytofluorometry according to the standard technique with immunophenotyping of T- and B-lymphocytes was performed.*
Results and discussion. *Compared to controls, patients in group 1 who had not previously received sDMARDs showed a transient increase in "switched" memory B-cells, transient B-cells, and plasmablasts, which was not observed in patients of group 2 (on sDMARDs therapy). Patients with advanced RA showed a statistically significant decrease in the absolute and relative number of memory B-cells, the absolute and relative number of "switched" B-lymphocytes, as well as the number of plasmablasts and transient cells. In RA patients, a statistically significant relationship was established between the number of swollen joints and the level of plasmablasts (r=0.51), memory cells (r=0.54), and "switched" B-cells (r=0.41), p<0,05 in all cases. There were no statistically significant changes in other subpopulations of B-lymphocytes and the profile of T-lymphocytes.*
Conclusion. *Changes in the B-lymphocyte profile are characteristic of different stages of RA. At an early stage, there is an increase in the number of transient B-lymphocytes, plasmablasts and plasmocytes, and in the advanced stage, a decrease in the level of certain populations of B-lymphocytes, such as memory B-cells and "switched" B-lymphocytes. It can be assumed that the ineffectiveness of sDMARDs is associated with a change in the population composition of B-lymphocytes, which requires further study.*

Key words: *rheumatoid arthritis; immunogram; methotrexate; CD19+ B-cells; Memory B-cells; naive B-cells; double negative B-cells; flow cytofluorometry.*
Contact: *Tatyana Valentinivna Popkova;* ***popkovatv@mail.ru***
For reference: *Martynova AV, Popkova TV, Aleksankin AP, et al. T- and B-lymphocytes subpopulations in the early and advanced rheumatoid arthritis. Sovremennaya Revmatologiya=Modern Rheumatology Journal. 2021;15(2):17–22.* ***DOI:*** *10.14412/1996-7012-2021-2-17-22*

Rheumatoid arthritis (RA) is an autoimmune rheumatic disease which is characterized by progressive damage to joints and internal organs due to massive disruption of both humoral and cell-mediated immunity [1]. RA progression depends not only on pro-inflammatory cytokines, produced by fibroblasts, macrophages, dendric cells, T-lymphocytes and others, but on antibody production by B-cells. Massive pathological signaling to autoimmune inflammation is provided by IgG, IgA and their complexes. Direct destructive action of antibodies to cyclic citrullinated peptide (ACCP) and antibodies to modified citrullinated vimentin (AMCV) in patients with early serum-positive RA is well known [2. 3]. Production of antibodies to citrullinated peptides is associated with pathological activation of B-cells. Massive production of rheumatoid factors (RFs) which occurs in 80% of RA patients leads to activation of the system of complement and is directly related to RA activity [4], causing immediate cell damage. Formation of ectopic germinative centers in the proximity of the damaged joints stimulates B-cell differentiation and a subsequent increase in RA activity [5; 6]. Simultaneously, B-cells perform antigen-presenting functions which causes disturbance in self-regulation and T-cell activation followed by the cytokine cascade [7].

Objective. To evaluate dynamics of T and B lymphocyte subsets in different stages of rheumatoid arthritis (RA)

Patients and methods. Fifty three patients with verified RA were included in the study (ACR/EULAR 2010 criteria). The mean age of the participants was 54.2 [47; 62] years. The first group consisted of 27 therapy na?ve patients (25 females, 2 males), the second group included 26 patients (22 females, 4 males) receiving synthetic basic anti-inflammatory drugs (sDMARDs) – methotrexate or leflunomide. The group characteristics are given in Table 1.

In the second group, 23 (88.5%) patients took methotrexate at a dose 20 [15; 20] mg/week; 11.5% received leflunomide 20 mg/day for a median period of 24 [6; 50] months. The control group included 29 individuals (23 females, 6 males), mean age 58.5 [53; 62] years. All patients signed the informed consent protocols.

For the study of T and B-lymphocytes we used venous blood from the cubical vein (2.7 mL), collected to vacuum EDTA-containing test-tubes (concentration 1.6 mg/mL, S-monovette, 2.7 mL K3E; Sarstedt, Germany). Immunophenotyping of B-lymphocytes of the peripheral blood included identification of the following subsets: B-cells (CD19+), all memory B-cells (CD19+CD27+), switched B-cells (CD19+IgD-CD27+) and

Table 1. Clinical characteristics of RA patients

Characteristics	Value	
	1st group (n=27)	2nd group (n=26)
Age, yrs, Me [25th;75th percentiles]	57.5 [49; 63]	53 [46; 65]
RA duration, months, Me [25th;75th percentiles]	6 [5; 12]	84 [24; 121]*
DAS28, Me [25th;75th percentiles]	5.8 [5.2; 6,2]	6.4 [5.8; 6.9]*
RF-positivity, n (%)	20 (74)	23 (88,5)
ACCP-positivity, n (%)	11 (41)	23 (88.5)*
Steroid therapy, n (%)	–	18 (69)
Non-steroidal anti-inflammatory drug therapy, n (%)	24 (89)	17 (65)
Methotrexate therapy duration, months, Me [25th;75th percentiles]	–	24 [7; 50]
Mediane methotrexate dose, mg/week Me [25th;75th percentiles]	–	20 [15; 20]
Tender joints count, Me [25th;75th percentiles]	8 [5; 8]	11 [8; 18]*
Swollen joint count, Me [25th;75th percentiles]	4 [3; 6]	12 [6; 16]*

* group difference at $p<0.05$

non-switched (CD19+IgD+CD27+) memory B-cells, naive (CD19+IgD+CD27-), double negative (CD19+IgD-CD27-) and transitional B-cells (CD19+IgD+CD10+CD38++CD27-), plasmocytes (CD19+CD38+) and plasmoblasts (CD19+CD38+++IgD-CD27+CD20-). The method of multicolor flow cytometry was used. The same was done for T-cell subpopulations: T-cells (CD3+), T-helpers (Th, CD3+CD4+), cytotoxic T-cells (Tc, CD3+CD8+), index of T-helpers to cytotoxic T-cells (Th/Tc) and natural killers (NK, CD3-CD56+).

Conjugated mouse monoclonal antibodies were used: CD19-ECD (R Phycoe- rythrin-TexasRed®-X, IgG1);CD45-PC7 (R Phycoerythrin Cyanin 7, IgG1), CD38- PC5 (R Phycoerythrin Cyanin 5.1, IgG1); CD20-PC5 (Beckman Coulter, USA); CD10-PE (IgG1, HI10a), CD27-PE (IgG1, MT271, Becton Dickinson, USA); human antibodies: IgD-FITC (Fluorescein Isothiocyanate, IA62, Becton Dickinson, USA). Isotypic (negative) control was used to determine the boundaries of non-specific B-cell conjugation to antibodies using Simultest IMK Plus Kit (CD45-FITC, CD14-PE, CD3-FITC, CD19-PE, CD4-FITC, CD8-PE) and IgG1-FITC, IGG2a-PE (Becton Dickinson, USA). Two polypropylene test-tubes were used for each patient (Coulter 12×75 мм, Beckman Coulter, USA). Five mcL of labelled antibodies was added to 50 mcL (1×10^6 cells) of blood sample and put in a dark place at room temperature for 15 minutes. After the 15-minute incubation, erythrocytes were lysed using IOTest 3 Lysing Solution (Beckman Coulter, USA). Fifty mcL of Flow-Count™ Fluorospheres was added to the obtained lymphocyte suspension, and the results of five-color dying of lymphocytes were assessed using Navios analyzer. For each blood sample 50 thousand events were counted. Cell populations were identified with CXP software (Beckman Coulter, USA).

C-reactive protein (CRP) and IgM RF levels were measured by immunonephelometry using BN Pro Spec (Siemens, Germany) and a commercial kit from Axis-Shield (UK). ACCP was determined by IFA-reaction. Normal values for CRP were considered to be lower than 5 mg/L, for RF <15 ME/mL, for ACCP <20 ME/mL.

Statistic analysis was performed with Statistica 8.0 (StatSoftInc., USA) using Mann–Whitney U test, presented as median (Me) and interquartile range (25% to 75%). Differences were considered statistically significant at $p<0.05$.

Results. Patients in both groups had high RA activity. In the 1st group CRP levels were 24 [8.6; 77] mg/L, DAS28 score – 5.8 [5.15; 6,2]; in the 2nd group – CRP levels – 40 [15.5; 72.9] mg/L and DAS28 score – 6.31 [5.64; 6.88].

Compared with healthy controls, patients in both groups showed lower absolute B-cell count, while total leucocyte levels were normal (1st group – 6.3 [5.9; 8.0] $\times10^9$/L, 2nd group – 7.6 [6.2; 9.3] $\times10^9$/L). B-cell subpopulation distribution is presented in Table 2.

Statistically significant correlation between the count of swollen joints and plasmoblasts (r=0.51), memory B-cells (r=0.54), and switched B-cells (r=0.41) was found ($p<0.05$ in all cases).

In the 1st group we observed elevated levels of switched B-cells (CD19+CD27+IgD-), transitional B-cells (CD19+CD38++CD10+IgD+CD27-), plasmoblasts (CD19+CD38+++CD27+IgD-CD20-), parallel to a decrease in the absolute B-cell count ($p<0.05$). A significant rise in plasmocyte levels (CD19+CD38+) was also found in the treatment naive patients.

In the 2nd group of patients with advanced RA, we found a statistically significant decrease in absolute and relative amounts of memory B-cells (CD19+CD27+), switched B-cells, (CD19+CD27+IgD-). A decrease in the amount of plasmoblasts (CD19+CD38+++ CD27+ IgD-CD20) and transitional B-cells (CD19+CD38++CD10+IgD+CD27-) significantly correlated with a longer duration of RA.

No statistically significant changes were noted in naive (CD19+CD27-IgD+), double negative (CD19+CD27-IgD-) or non-switched B-cells (CD19+CD27+IgD+). When comparing two groups of RA patients, we found lower relative values of all B-lymphocyte populations in patients with advanced RA than with early RA, except non-switched and na?ve B-cells .

ACCP-positive patients with early RA statistically more often had increased levels of non-switched B-cell

Table 2. B-cell subpopulations in RA patients

B-cell subsets	1st group (n=27)		2nd group (n=26)		controls (n=29)	
	Absolute, 10^9/L	Relative, %	Abs., 10^9/L	Rel.,%	Abs., 10^9/L	Rel.,%
B-lymphocytes (CD19+)	0,144 [0,1; 0,21]	9,1 [7,5; 10,9]	0,105 [0,1; 0,2]**	8,35 [6; 10,2]	0,2 [0,1; 0,2]	8,5 [7,2; 11,0]
Memory B-cells (CD19+CD27+)	0,003 [0,00166; 0,0044]	2,1 [1,6; 3,1]	0,0015 [0,001; 0,003]*	1,25 [0,9; 1,7]*,**	0,003 [0,001; 0,007]	2,2 [1,1; 3,0]
Switched B-cells (CD19+IgD-CD27+)	0,0187 [0,0133; 0,0289]	16 [9,3; 18,4]	0,01 [0,005; 0,02]	6,8 [3,6; 11,6]*,**	0,02 [0,0; 0,04]	12,8 [9,3; 17,0]
Non-switched B-cells (CD19+IgD+CD27+)	0,0073 [0,00619; 0,0122]	5,9 [3,6; 9,7]	0,009 [0,006; 0,01]	7,45 [5,1; 11,4]**	0,01 [0,005; 0,02]	7,4 [3,7; 11,1]
Double-negative B-cells (CD19+IgD-CD27-)	0,021 [0,011; 0,028]	14 [9,6; 19,5]	0,02 [0,01; 0,03]	15,05 [11,9; 18,1]	0,02 [0,01; 0,02]	13,3 [7,1; 19,3]
Naive B-cells (CD19+IgD+CD27-)	0,076 [0,063; 0,13]	61,6 [52,9; 68,8]	0,095 [0,07; 0,1]	70,85 [62,5; 75,6]**	0,1 [0,06; 0,1]	64,7 [57,6; 72,4]
Transitional B-cells (CD19+IgD+CD10+ CD38++CD27-)	0,000424 [0,000162; 0,000624]*,**	0,2 [0,1; 0,4]	0 [0; 0,0001]	0 [0; 0,1]	0,0001 [0; 0,0003]	0,1 [0; 0,1]
Plasmoblasts (CD19+CD38+++ IgD-CD27+CD20-)	0,00071 [0,00023; 0,00129]*,**	0,4 [0,3; 0,8]*,**	0,0003 [0,00007; 0,0004]	0,15 [0,1; 0,3]	0,0002 [0,0001; 0,0004]	0,1 [0,1; 0, 2]
Plasmocytes (CD19+CD38+)	0,000262 [0,000106; 0,000414]*,**	0,1 [0,1; 0,3]	0,0001 [0; 0,0002]	0,1 [0; 0,1]	0,0001 [0,00; 0,0002]	0,1 [0,05; 0,1]

* – difference between a group and controls ($p<0.05$); ** – difference between groups ($p<0.05$).

(CD19+CD27+IgD+) and absolute number of transitional B-cells (CD19+CD38++CD10+IgD+CD27-): 0,0237 vs 0,007 ($p<0,05$) and 0,00079 vs 0,00027 ($p<0,02$), respectively. No other significant correlations were discovered.

No differences in T-cell subsets were found (Fig.1).

Discussion. It is well known that in RA pathogenesis an important role is played by antibody-dependent (production of ACCPs, IgG, IgA of RF) and antibody-independent (pro-inflammatory cytokines, T-cell activated tissue inflammation) reactions [7, 8]. Gene activation and interleukin-2 production are followed by T-cell proliferation which causes antigen-specific T-cell spawn, including CD8+ T-cells. It causes antigen-carrying target cell elimination and production of pro-inflammatory cytokines by CD4+ T-cells, and, therefore, activation of local tissue lymphocytes and macrophages [9]. This cascade of reactions causes T-cell migration to the inflamed joint. Lack of T-cell production or changes in their subsets in our study may indicate a very limited cytokine response in the individuals with high RA activity and the prevalence of humoral response. At the same time, the observed tendency to elevation of cytotoxic T-cells indicates hyperproduction of tumor necrosis factor-α (TNFα) and interferons γ (γIF). Thus, the existing data suggest local intra-articular activation of regulatory T-lymphocytes and a subsequent increase in the levels of γIF, TNFα, IL9, IL10, etc. [10–14]. We did not investigate T-helper subsets, but their differentiation and activation of different subtypes may be the basis of cytokine reaction. In addition, intra-articular increase in some T-lymphocyte subtypes in ectopic germinative sites [5, 6] cannot be excluded, which may be a likely reason for the lack of extra-articular signs in the observed RA patients.

Humoral immunity activation is common before the manifestation of the disease or in its very early stage. Rising levels of ACCP and RF were reported in individuals with a high risk of RA development [2, 3]. The basis of inflammation is pathological B-cell activation in response to T-cell presence and local pro-inflammatory factors. This leads to massive immunoglobulin production and autoactivation of other T- and B-cell subsets. In a review by S. Bugatti et al. [15] negative changes in T-cell functions and subsequent dysregulation of the production and apoptosis of B-lymphocytes were observed. Increased production of serum B-cell activation factor by such B-lymphocytes results in increased levels of B-cells and their uncontrolled maturation. In our study we observed no changes in general B-cell levels, yet a substantial decrease in memory B-cells, transitional and switched B-lymphocytes, and plasmoblasts was evident in both groups of patients, unlike in healthy controls.

Therefore, due to the disturbance in maturation and apoptosis of B-cells and their migration into the joint, lower levels of absolute and relative count of switched B-cells (CD19+CD27+IgD-), transitional B-cells (CD19+CD38++CD10+ IgD+CD27-), plas-

moblasts (CD19+CD38+++CD27+ IgD-CD20-) and plasmocytes (CD19+ CD38+) were observed in advanced RA group; the same cells were increased in the therapy naive patients, compared with healthy controls. This may be explained by intra-articular B-cell migration and further local ACCP production, which is confirmed by higher rates of ACCP and RF positivity in the 2nd group.

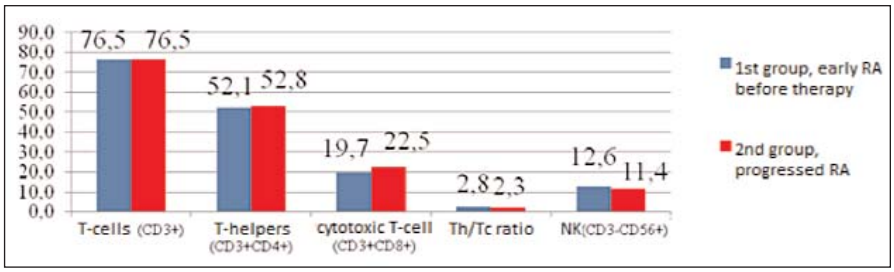

Figure 1. *Subsets of T-lymphocytes, relative numbers, %*

At the same time, we did not find any changes in non-switched B-cell (CD19+CD27+IgD+) levels in advanced RA group, compared with controls, despite decreased levels of these cells in the treatment naive patients in the 1st group. It contradicts the data of F. Hu et al. [16], who found a decrease in this subset in RA patients, but their patients received TNFα-inhibitors and methotrexate. They concluded that the observed changes may be connected with impaired function of these lymphocytes, which was also noted in our work. The same conclusions, along with differences in surface antigen production, were made by Y. Wang et al. [17], who analyzed the data from two large RA cohorts (n=3494 and n=397). They described a significant rise in IgM and IgA levels in blood serum with no changes in B-cell subsets, along with an increase in ACCP levels in serum-positive patients which was connected with increased levels of double-negative B-cells (CD19+CD27-IgD-). In the work of R.A. Moura et al [18] the patients before RA therapy (n=13) and the patients on methotrexate (n=20) also had increased levels of double-negative B-cells.

According to our data, there is a tendency to an increase in double-negative B-cells in patients with high activity and longer duration of RA. Interestingly, the growth of this subpopulation might be due to depletion of memory B-cell pool and inflammatory damage to B-cells, which is yet to be investigated [16]. S. Nakayamada et al. [19] found decreased memory B-cell levels in the peripheral blood samples of RA patients, compared with healthy individuals. This is consistent with the data obtained by J. Lü bbers et al. [20] who observed a decrease in CD27+ memory B-cells in all 89 patients with RA (naive to therapy), compared with healthy donors, but without any changes in other B-cell subsets. In our study patients in the 1st group, naive to therapy, initially had the same levels of memory B-cells as healthy controls. It may indicate initial humoral response to autoinflammation while continuous inflammation causes down-regulation of memory B-cells. Interestingly, the patients with advanced RA were significantly more often ACCP- and RF- positive than those with an early stage. In the study by M.M. Souto-Carneiro et al. [21] in patients on TNFα-inhibitor therapy, memory B-cell levels did not show any dynamics, which may be explained by effective RA-activity control. Additionally, a certain pool of B-cells, in particular memory B-cells resistant to therapy, may persist in patients with no response to CD20 blockers [22–24]. It cannot be excluded, that in our study resistance to therapy in the 2nd group was a sign of lost efficiency of the therapy due to incomplete depletion of certain groups of CD27+ B-cells, memory B-cells in particular [25].

In our study a linear direct association was established between the number of tender joints and levels of plasmoblasts (r=0.51), memory B-cells (r=0.54) and switched B-cells (r=0.41), which is an expected result of end-stage B-cell differentiation. No other clinical and laboratory associations of B- and T-lymphocytes in RA were established.

Conclusion. Changes in B and T-cell subset levels are common in different stages of RA. Characteristically, in the early stage the levels of transitional B-cells, plasmoblasts and plasmocytes are elevated, while in the advanced stage of RA a decrease in levels of memory B-cells and switched B-cells is common. It can be suggested that inefficacy of sDMARDs might correlate with changes in B-cell subsets, which requires further research.

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Received/Reviewed/Accepted
29.01.2020/3.03.2021/7.03.2021

The research was carried out with the financial support of the Russian Foundation for Basic Research within scientific project № 19-315-90090.

The investigation has not been sponsored. There are no conflicts of interest. The authors are solely responsible for submitting the final version of the manuscript for publication. All the authors have participated in developing the concept of the article and in writing the manuscript. The final version of the manuscript has been approved by all the authors.

Martynova A.V. https://orcid.org/0000-0001-6840-4552
Popkova T.V. https://orcid.org/0000-0001-5793-4689
Aleksankin A.P. https://orcid.org/0000-0001-6686-0896
Gridneva G.I. https://orcid.org/0000-0002-0928-3911
Gerasimova E.V. https://orcid.org/ 0000-0001-5815-561X
Gorbunova Yu.N. https://orcid.org/0000-0002-2024-6927
Lila A.M. https://orcid.org/0000-0002-6068-3080