Disease activity in patients with long-lasting rheumatoid arthritis is associated with changes in peripheral blood lymphocyte subpopulations

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ABSTRACT

INTRODUCTION Rheumatoid arthritis (RA) is a chronic autoimmune disease and it is known that lymphocytes play a major role in its pathogenesis. However, there have been no comprehensive studies on the changes in peripheral blood lymphocyte (PBL) subpopulations expressing different clusters of differentiation (CD) in patients with long-lasting RA.

OBJECTIVES The aim of our study was to measure the main subpopulations of PBL, expression of costimulatory marker CD28, and activation status of CD4⁺ T cells depending on clinical disease activity in long-lasting RA.

PATIENTS AND METHODS The study comprised 60 patients with RA and 19 healthy volunteers. Disease activity, the proportion and number of the main PBL subpopulations (T, B, natural killer [NK], and NK T cells [NKT]), the expression of costimulatory marker CD28, and the activation status of CD4⁺ T cells were evaluated on the same day. A multicolor flow cytometry with marked monoclonal antibodies was used for the assessment of lymphocyte subpopulations.

RESULTS The percentage of CD3⁺CD4⁺, NKT, CD4⁺CD28⁻, CD8⁺CD28⁻, CD4⁺CD69⁺, CD4⁺CD25⁺, and CD4⁺HLA-DR⁺ was significantly higher in RA compared with the control group. A higher proportion of CD4⁺CD28⁻ was associated with more active disease, while an inverse correlation was observed for B cells. The proportion of CD4⁺CD28⁻ was not associated with disease activity. The number of CD4⁺CD69⁺ cells in RA patients increased with increasing DAS28, while the number of CD4⁺HLA-DR⁺ T cells showed no such association.

CONCLUSION Our results have shown for the first time an association between the phenotype patterns of PBL T, B, and NKT and RA activity in patients with long-lasting disease, which reinforces the hypothesis that PBL play an important role in modifying or maintaining the disease activity.

KEY WORDS activation marker, CD28, disease activity, peripheral blood lymphocytes, rheumatoid arthritis

INTRODUCTION Rheumatoid arthritis (RA) is a chronic inflammatory and autoimmune disease, which is not limited to the joints but is also associated with systemic symptoms of inflammation, including increased erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and shift of cytokine balance towards proinflammatory mediators also in the peripheral blood.¹ RA activity measured by the disease activity score in 28 joints (DAS28) reflects both the presence of local arthritis measured by the number of swollen and tender joints and laboratory findings indicating systemic inflammation, such as ESR or CRP.² The involvement of the immune system in RA pathogenesis is not limited to inflammation. T and B cells play an important role in disease progression, as shown by the studies on synovial fluid or synovial membrane lymphocyte subpopulations identified according to the cluster of differentiation (CD) markers.³ ⁴ The involvement of peripheral blood T cells in RA pathogenesis has been studied mainly in the context of altered CD28 expression on CD4⁺ and CD8⁺ T cells in patients with RA.⁵ ⁶ Recently, anti-B therapy has confirmed that B cells are involved in local synovitis.⁷
Activated lymphocytes are known to express a number of surface molecules, including CD69, CD25, and HLA-DR, which are expressed in small amounts or not at all on the resting cells. There is limited knowledge on the association between the number and activation status of CD4+ T cells in the peripheral blood of RA patients. The available studies focused on erosive RA, the effects of methotrexate (MTX) treatment,13,16 or the age of patients at RA onset.11

Data from these studies support the concept of the major role of lymphocytes in immune-mediated RA. However, changes in the adaptive immune system, including all major peripheral blood subpopulations, activation markers of CD4+ T cells, and CD28 expression on T cells and their association with disease activity, have not been analyzed so far in a single study. We decided to examine the cellular adaptive immune system in long-lasting RA and to measure the percentage and number of different lymphocyte subpopulations in the peripheral blood of RA patients with different disease activity.

Patients and Methods Patients It was an immunological study involving 60 patients (53 women and 7 men) with long-lasting advanced RA. Patients fulfilled at least 4 criteria of the American College of Rheumatology.12 Duration of RA was at least 1 year. All patients were on low doses of MTX (7.5–15 mg/wk) and/or of prednisone therapy (<10 mg/day) for the minimum of 6 months, according to a therapeutic protocol described by Bijlsma.13 Patients receiving any type of biological treatment were excluded from the study. Patients with other connective tissue or autoimmune diseases, kidney diseases, hepatitis or other serious liver diseases were also excluded based on clinical examination and typical screening tests (hepatitis C virus antibody, hepatitis B surface antigen, antinuclear antibodies, alanine and aspartate transaminases [normal ranges required because of MTX treatment], and glomerular filtration rate). To eliminate yet another confounding variable, only patients positive for the rheumatoid factor were included in the study.

Patients were divided into 3 groups according to the European League Against Arthritis criteria based on DAS28: group 1 with low disease activity (DAS28 ≤3.2); group 2 with moderate disease activity (3.2 < DAS28 ≤5.1); and group 3 with severe disease activity (DAS28 >5.1). The clinical characteristics and basic laboratory tests in RA patients divided based on DAS28 are presented in Table 1.

To obtain the cutoff points with the aim of comparing the differences between lymphocyte subpopulations, we recruited 19 healthy subjects (15 women and 4 men; mean age, 45.2 ±19.4 years).

All individuals included in the study gave their written informed consent and the Bioethical Committee of the Medical University of Gdańsk approved the study.

Clinical assessment Venous blood was obtained for the analysis of ESR and lymphocyte subpopulations. On the same day, the clinical parameters (28 swollen [Sw] and tender [Ten] joint counts) and the functional status (visual analogue scale – VAS) were assessed. DAS28 was calculated for each patient according to the following formula: DAS28 = 0.56 × √Ten28 + 0.28 × √Sw28 + 0.7 × In(ESR) + 0.014 × VAS. The radiographic status was estimated by the Steinbrocker method.

Cytometric analyses of lymphocyte subpopulations Samples of peripheral blood (100 ml) were stained for 30 minutes on ice with fluorochrome-conjugated antibodies (anti-): CD3-FITC, CD4-RPE-Cy5, CD8-RPE-Cy5, CD69-PE, CD25-PE, CD28-PE, HLA-DR-PE, CD19-RPE-Cy5, CD16+56+PE (surface markers and isotype control: anti-CD3, CD4, CD8, and CD19 from Dako, Denmark; the rest of antibodies from BD Bioscience, United States). Antibody concentrations were adjusted for the optimal staining. Red blood cells were lysed by the cell lysis buffer for 15 minutes at room temperature. Samples were assayed using a multicolor flow cytometry (FACScan, Becton Dickinson, California, United States) and the WinMDI 2.9 software for data analysis. Lymphocytes were identified by forward- and side-scattered light gating, and the percentage of B cells, natural killer (NK) cells and NK T cells (NKT) were calculated in peripheral blood lymphocytes (PBL). For the analysis of activation markers on CD4+ T cells, T cells were gated based on their CD3 and CD4 expression, and the expression of activation antigens was analyzed only for CD3-CD4- T cells. Regarding CD28 expression on CD4+ and CD8+ T cells, we applied gating strategy used in our previous studies.14,15 To distinguish activated from regulatory lymphocytes among CD4+CD25+ T cells, the gating strategy was used according to a recently described method, in which CD4+<CD25<0.01 T cells were classified as regulatory T cells based on their expression of Foxp3 marker and functional tests,16 and CD4+CD25+ were considered to be activated CD4+ T cells.

Statistical analysis A statistical analysis was performed using the Statistica 9 StatSoft software. The Kolmogorov-Smirnov test was used for distribution analysis. The differences in the percentages of peripheral blood T cells between the groups were assessed using the analysis of variance and the post-hoc Scheffé test. In the case of nonsymmetrical distribution of the variables, the Kruskal-Wallis and Mann-Whitney U tests were applied.

Results Characteristics of patients depending on disease activity There were no significant differences in age, disease duration, or a dose of MTX or prednisone between the groups of patients with different disease activity measured by DAS28. The only differences were observed for the Steinbrocker stage and ESR: patients with the most
active RA had more advanced radiological changes and the highest ESR compared with those with the lowest disease activity (TABLE 1).

**TABLE 1** Clinical characteristics and basic laboratory data of patients with rheumatoid arthritis with different DAS28

<table>
<thead>
<tr>
<th>Variables</th>
<th>DAS28 ≤3.2 n = 10</th>
<th>3.2 &lt; DAS28 ≤5.1 n = 36</th>
<th>DAS28 &gt;5.1 n = 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>age, y</td>
<td>40 ±13</td>
<td>45 ±17</td>
<td>47 ±16</td>
</tr>
<tr>
<td>sex, men/women</td>
<td>2/8</td>
<td>3/33</td>
<td>3/11</td>
</tr>
<tr>
<td>disease duration, y</td>
<td>3 (1.5; 4)</td>
<td>5 (2.5; 7)</td>
<td>2.5 (2.5; 7)</td>
</tr>
<tr>
<td>Steinbrocker score</td>
<td>2 (1; 2)</td>
<td>2 (1; 3)</td>
<td>3 (2; 4)</td>
</tr>
<tr>
<td>ACR criteria, % fulfilling 4/5/6/7 criteria</td>
<td>33/17/33/17</td>
<td>16/19/36/29</td>
<td>9/10/43/38</td>
</tr>
<tr>
<td>other comorbidities, % of patients</td>
<td>30</td>
<td>33</td>
<td>43</td>
</tr>
<tr>
<td>bronchial asthma</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>gastritis</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>essential hypertension</td>
<td>1</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>diabetes (after treatment)</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>cholelithiasis</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>otitis media</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>uveitis</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>osteoporosis</td>
<td>0</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>MTX dose, mg/vk</td>
<td>12.25 (10.0; 15.0)</td>
<td>10.0 (10.0; 12.5)</td>
<td>10.0 (10.0; 12.5)</td>
</tr>
<tr>
<td>prednisone dose, mg/d</td>
<td>6.0 (2.5; 10.0)</td>
<td>7.5 (5.0; 10.0)</td>
<td>10.0 (5.0; 10.0)</td>
</tr>
<tr>
<td>ESR, mm/1 h</td>
<td>10.15 ±3.15*</td>
<td>20.90 ±10.93*</td>
<td>63.71 ±29.97*</td>
</tr>
<tr>
<td>positive RF, % of patients</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>WBC count, G/l</td>
<td>6.33 ±2.19</td>
<td>7.70 ±2.46</td>
<td>8.21 ±2.72</td>
</tr>
<tr>
<td>lymphocytes, %</td>
<td>32.47 ±10.25</td>
<td>29.28 ±5.83</td>
<td>27.40 ±8.40</td>
</tr>
<tr>
<td>lymphocytes, G/l</td>
<td>2.05 ±0.22</td>
<td>2.25 ±0.14</td>
<td>2.25 ±0.23</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD or median (25th and 75th quartile).

* P <0.05

Abbreviations: ACR – American College of Rheumatology, DAS28 – disease activity score in 28 joints, ESR – erythrocyte sedimentation rate, MTX – methotrexate, RA – rheumatoid arthritis, RF – rheumatoid factor, SD – standard deviation, WBC – white blood cell

Percentage and absolute number of lymphocyte subpopulations depending on DAS28 Patients with RA have an increased percentage of CD3+CD4+ T cells compared with the control group; on the other hand, there were no significant differences between RA patients divided according to DAS28. The percentage of CD3+CD8+ cells in the peripheral blood from the RA and control groups were similar. Moreover, no significant differences were found between RA patients with different disease activity (TABLE 2).

We observed a trend towards increasing percentage and absolute number of NKT (CD3+CD16+CD56+) cells with increasing DAS28 value for RA patients and increased percentage of NKT cells in the RA group compared with the control group.

Finally, we observed significant differences only in the number and not in the percentage of B (CD19+) lymphocytes between patients divided according to DAS28. Subjects with severe disease activity were characterized by a lower number of B cells than those with low disease activity, but the numbers were comparable to those observed in the control group.

All patients with RA had higher percentage and absolute number of peripheral blood CD4+CD28- and CD8+CD28- cells compared with the control group. However, the values differed depending on disease activity. The percentage and absolute number of CD4+CD28- cells (but not of CD8+CD28-) cells was observed to increase with increasing DAS28 (FIGURE 1).

CD4+CD69+ cells in the peripheral blood were activated earlier in patients with RA compared with healthy controls. Moreover, the number of CD4+CD69+ cells in RA patients increased with increasing DAS28 (FIGURE 2A).

The percentage of activated CD4+CD25+ T cells in the peripheral blood was higher in patients with RA compared with controls, but only in those with medium and high disease activity (FIGURE 2CD).

All patients had a higher percentage and absolute number of CD4+HLA-DR+ T cells compared with the control group (FIGURE 2E).

**DISCUSSION** The aim of our study was to investigate whether the percentage and absolute number of major lymphocyte subpopulations,
suggesting a role of these lymphocytes in RA pathogenesis, we may only suggest that this involvement might depend on qualitative and not quantitative changes in this population.

In a study by Masuko-Hongo et al., which showed the expansion of CD3+CD8+ cells in the synovial fluid, patients with RA had different disease activity but they were not divided according to DAS28, so the comparison with our results is difficult. Although the increased percentage of CD3+CD8+ T cells in the synovial fluid suggests an important role of these cells in the local area of the joints in RA, the fact that we did not show any decrease or increase in the number of CD3+CD8+ T cells in the peripheral blood of RA patients with long-lasting RA change depending on disease activity compared with healthy individuals.

In line with the previous studies, we showed an increase in the percentage of CD3+CD4+ cells in RA patients compared with the control group. Additionally, we have demonstrated for the first time that patients with moderate disease activity have the highest percentage of CD3+CD4+ cells in the peripheral blood compared with patients with other disease activity and the control group. We did not find similar associations in the number and percentage of CD3+CD8+ T cells. In light of the reports suggesting a role of these lymphocytes in RA pathogenesis, we may only suggest that this involvement might depend on qualitative and not quantitative changes in this population. In a study by Masuko-Hongo et al., which showed the expansion of CD3+CD8+ cells in the synovial fluid, patients with RA had different disease activity but they were not divided according to DAS28, so the comparison with our results is difficult. Although the increased percentage of CD3+CD8+ T cells in the synovial fluid suggests an important role of these cells in the local area of the joints in RA, the fact that we did not show any decrease or increase in the number of CD3+CD8+ T cells in the peripheral blood of RA patients with long-lasting RA change depending on disease activity compared with healthy individuals.

### Table 2: Percentage and absolute number of lymphocyte subpopulations in patients with different DAS28 and healthy controls

<table>
<thead>
<tr>
<th>Lymphocytes</th>
<th>Controls</th>
<th>DAS28 ≤3.2</th>
<th>3.2 &lt; DAS28 ≤5.1</th>
<th>DAS28 &gt;5.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3+CD4+, %</td>
<td>43.50 ±8.6a</td>
<td>47.0 ±8.1</td>
<td>53.5 ±10a</td>
<td>46.7 ±9.4</td>
</tr>
<tr>
<td>CD3+CD4+, G/l</td>
<td>1.0 ±0.2</td>
<td>1.0 ±0.4</td>
<td>1.1 ±0.5</td>
<td>0.8 ±0.2</td>
</tr>
<tr>
<td>CD3+CD8+, %</td>
<td>20 ±8.7</td>
<td>16.5 ±8.5</td>
<td>22.5 ±6.1</td>
<td>19.8 ±6.1</td>
</tr>
<tr>
<td>CD3+CD8+, G/l</td>
<td>0.5 ±0.2</td>
<td>0.25 ±0.2</td>
<td>0.43 ±0.23</td>
<td>0.38 ±0.015</td>
</tr>
<tr>
<td>CD3+CD16+CD56+, %</td>
<td>0.85 ±0.72a</td>
<td>1.5 ±0.9</td>
<td>2.85 ±1.8a</td>
<td>3.4 ±1.6</td>
</tr>
<tr>
<td>CD3+CD16+CD56+, G/l</td>
<td>0.02 ±0.01a</td>
<td>0.034 ±0.015</td>
<td>0.06 ±0.03a</td>
<td>0.025 ±0.015</td>
</tr>
<tr>
<td>CD19+, %</td>
<td>7.2 ±4.2</td>
<td>11.3 ±4.8</td>
<td>7.6 ±5.2</td>
<td>7.4 ±5.4</td>
</tr>
<tr>
<td>CD19+, G/l</td>
<td>0.14 ±0.05</td>
<td>0.19 ±0.13a</td>
<td>0.15 ±0.06</td>
<td>0.12 ±0.07a</td>
</tr>
</tbody>
</table>

a P <0.05

Percentage of the main lymphocyte subpopulations was calculated as the percentage of peripheral blood lymphocytes; absolute numbers were calculated based on lymphocyte count in the peripheral blood and percentage of every subpopulation.

**Abbreviations:** see **Table 1**

**Figure 1** Mean percentage (A, C) and absolute number ±1.96 standard deviation (B, D) of peripheral blood lymphocytes with different cluster of differentiation (CD) expression: CD4+CD28− (A, B) and CD8+CD28− lymphocytes (C, D) in controls and patients with rheumatoid arthritis divided according to disease activity score 28 (DAS28); the percentage of CD28− and CD28+ CD4+ and CD8+ T cells was calculated separately for CD4+ and CD8+ T cells, and summarized to 100% for every type of T cell. a – statistically significant.
patients compared with the control group could, in our opinion, indicate that those cells proliferate locally and remain in rather than migrate between synovial tissues and the bloodstream.

We demonstrated a trend towards an increase in the percentage and number of NKT cells with increasing disease activity. It is an interesting finding because currently there are only a few preliminary reports about NKT cells in the peripheral blood of RA patients. The available data are not consistent. Some investigators did not show any differences in the percentage of CD3⁺CD56⁺ cells between RA patients and healthy controls,²⁰ while others observed a higher proportion of NKT (CD3⁺CD56⁺) cells in RA patients (but only in women),²¹ which is in agreement to some extent with our results because most of our RA patients were women. None of the above studies divided RA patients into groups based on disease activity.

The significance of an increase in the percentage and number of NKT cells is still unknown. Possibly, these cells might participate in sustaining an increased inflammatory status in patients (translating to higher disease activity), because they are able to produce high levels of proinflammatory cytokines and show cytotoxic ability. On the other hand, they also produce anti-inflammatory cytokines, which can shift the balance towards T helper (Th) 2 response and increase antibody production.²² It was shown that in patients with systemic lupus erythematosus, the increased proportion of NKT cells was associated with higher anti-immunoglobulin G anti-DNA antibodies.²¹ The significance of NKT cells in various diseases is elusive because they play a dual role: they control some diseases (diabetes) while exacerbating others (lupus, atherosclerosis).²²

In our study, we showed an association between a decreased number of CD19⁺ B cells and increased DAS28. In light of the results confirming the role of B cells in the pathogenesis of RA²³ and successful treatment,²⁴ this decrease seems to be biologically significant and could mean an increased migration of B cells into the synovium with increasing disease activity.²⁵ Such a possibility was postulated in numerous previous papers.²⁶,²⁷ Nonetheless, an appropriate approach to this issue requires either a clonotypic analysis of B cells present in the synovium and blood (and demonstration of differences in the proportions of specific clones) or animal model studies, in which tagged B cells would be reinjected to the blood of RA animal and then detected in its inflamed synovial tissue. There is only 1 paper that applied the first approach²⁸ and showed that B cells invading the synovial fluid might be the progeny of blood-roaming B-cell clones.

**FIGURE 2** Mean percentage (A, C, E) and absolute number (B, D, F) of activated CD4⁺CD69⁺, CD4⁺CD25⁺, and CD4⁺HLA-DR⁺ cells in controls and patients with rheumatoid arthritis divided according to disease activity score 28 (DAS28); the percentage of activated CD4⁺ T cells was calculated in CD4⁺ T-cell subset; the absolute number of activated CD4⁺ T-cell subpopulations were calculated based on the absolute lymphocyte count in peripheral blood and percentage of every subpopulation in lymphocyte gate. a – statistically significant.
There are conflicting data regarding the increased presence of CD4^+^CD28^−^ and CD8^+^CD28^−^ T cells in the peripheral blood of RA patients.\textsuperscript{30} In a number of studies, an increased percentage of T cells without CD28 was typical of all RA patients,\textsuperscript{31} but other studies found an increased representation of that subpopulation in RA patients with extraarticular disease\textsuperscript{32} or observed an association with more aggressive bone destruction.\textsuperscript{33} The role of these CD28^−^ T cells in the peripheral blood is not fully understood but RA patients with the expansion of these cells showed a decreased vasodilatation and significantly thicker intima-media thickness in the arteries, suggesting increased susceptibility to atherosclerosis and its complications.\textsuperscript{34} Our data showed that CD4^+^CD28^−^ and CD8^+^CD28^−^ T cells were increased in RA patients compared with healthy controls, and the percentage of CD8^+^CD28^−^ was higher than that of CD4^+^CD28^−^. The difference is not entirely clear, but it could result from the initially lower number of CD28 molecules on CD8^−^ than on CD4^+^ T cells in the peripheral blood both in healthy individuals and RA patients, and exposure to tumor necrosis factor (TNF) directly decreasing number of CD28 molecules on T cells\textsuperscript{35} could result in the prevalence of increased proportion of CD8^−^CD28^−^ T cells in RA patients.

Only an increase in CD4^+^CD28^−^ T cells was associated with disease activity, which in our opinion suggests a more direct involvement of those cells in disease activity regulation than that of CD8^+^CD28^−^ cells (which seem to be more important in local, or in-joint, disease activity.\textsuperscript{36} An increased percentage of CD4^+^ T cells without CD28 molecule in patients with RA is associated with increased disease activity, which suggests the involvement of these cells in the development of an inflammatory status. This, in turn, increases the risk for cardiovascular disease\textsuperscript{37} because those cells were found to be directly involved in coronary vessel injury and were shown to be cytotoxic.\textsuperscript{38} This could also be associated with increased inflammation in patients with higher values of DAS28 and with increased secretion of TNF, which was shown to influence directly the transcription of CD28 gene.\textsuperscript{39} On the other hand, the percentage of CD3^+^CD8^+^CD28^−^ T cells was increased in all RA patients and was not associated with disease activity, which could suggest different regulation of CD28 expression on those cells or their different migration ability.

RA patients showed increased proportions of activated CD4^+^T cells in the peripheral blood compared with controls. Moreover, the proportions differed to some extent between patients with different disease activity, but the pattern of these changes in association with DAS28 was different for various activation markers. In short, CD69^+^ and HLA-DR^+^ cells showed a trend towards increased values with higher RA activity, reaching statistical significance. The same trend could barely be observed for CD25^+^ lymphocytes.

Earlier studies concerning T-cell activation phenotypes in RA patients investigated mainly the synovial fluid, identifying T-cell subpopulations in the affected joints rather than in the periphery. It was shown that the expression of CD69 marker was the highest on CD3^+^ cells in the synovial fluid of RA patients compared with patients with seronegative arthritis or gout,\textsuperscript{4} and also in the synovial membrane in RA patients.\textsuperscript{40} Therefore, it is believed that CD3^+^CD69^+^ play an essential role in RA pathomechanism both in the local area of the joint and in the periphery. Recently, a new role for CD69 molecule has emerged as an inhibitor of Th17-cell differentiation, at least in CD69 knockout mouse,\textsuperscript{41} but also as a local regulator of tumor growth factor-β production.\textsuperscript{42} These results were obtained in mouse studies and in local synovitis, so the role of this subpopulation in human peripheral blood is still unknown. Our results on CD4^+^CD69^+^ lymphocytes seem to support the hypothesis that early activated T cells participate in the pathogenesis and activity of RA.

There are conflicting results on the changes in CD4^+^CD25^+^ T cells in RA patients. A number of studies did not show any differences between RA patients and controls,\textsuperscript{43} while others found a higher percentage of CD4^+^CD25^+^ cells in RA patients.\textsuperscript{44} These discrepancies may result from wrong distinction between CD25^+^ activated and CD25^+^ regulatory cells. In our study, we found that we counted only activated CD4^+^CD25^+^ cells and we observed no significant differences between RA patients with different disease activity.

Finally, we demonstrated a trend towards an increase in the CD4^+^HLA-DR^+^ subpopulation in the blood of RA patients compared with healthy controls. Interestingly, the percentage of CD4^+^ T cells expressing this late activation marker did not differ between RA patients with different disease activity. To summarize our findings on the expression of activity markers on CD4^+^ T cells, an increase in both early and late T-cell activation markers is associated with moderate-to-severe disease activity, which could be a result of exacerbation and increase in early activation markers overlaid on chronic activation.

The limitation of our study is lack of cytokine measurements in different lymphocyte subpopulations. The aim of this paper was to check different PBLs in a “native” status, which means without activation in vitro and in association with disease activity. In our opinion, cytokine measurement in different subpopulations requires a strong stimulation with mitogens or ionophores and reveals the maximum ability of cells to secrete cytokines. We believe that the information provided by this method could not be directly associated with the in-vivo status; therefore, we did not perform cytokine measurements in different lymphocyte subpopulations. On the other hand, the measurement of circulating cytokines in the serum of RA patients had already been published by numerous authors including ourselves.\textsuperscript{45} In our previous studies, we showed a strong correlation...
between serum interleukin (IL) 6 levels and disease activity measured by DAS28, and no correlation for TNF-α, IL-2, IL-10, and IL-4, and a negative correlation for interferon γ.12

The effect of treatment on lymphocyte subpopulations in the peripheral blood was maximally reduced as a factor in our patients due to a study design (including patients only with long-lasting RA, on stable doses of MTX and prednisone at least for 6 months, and receiving no other drugs known to directly affect lymphocyte subpopulations). In our opinion, this allowed us to associate the described changes in the peripheral blood of RA patients with disease activity and not with treatment.

In conclusion, our data seem to support the hypothesis about the indirect involvement of peripheral lymphocytes in the pathogenesis and course of RA by showing, for the first time, an association between the percentage of different phenotypes of T cells, B cells, and NKT cells and RA activity measured by DAS28, suggesting that PBL play an important role either in modifying or maintaining the disease activity.

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REFERENCES

4 Ozto AN, Leffon A, Gonzalez-Aviles I. CD99 expression on lymphocytes and interleukin-15 levels in synovial fluids from different inflammatory arthropathies. Rheumatol Int. 2002; 21: 192-188.
STRESZCZENIE

Reumatoidalne zapalenie stawów (RZS) jest przewlekłą chorobą autoimmunologiczną, w której patogenezie ważną rolę odgrywają limfocyty. Brakuje jednak kompleksowych badań dotyczących zmian subpopulacji limfocytów krwi obwodowej różniących się ekspresją markerów powierzchniowych (clusters of differentiation – CD) we krwi obwodowej u pacjentów z długotrwałym RZS.

CELE Celem pracy było zbadanie wszystkich głównych subpopulacji limfocytów, ekspresji cząsteczki kostymulującej CD28 oraz statusu aktywacyjnego limfocytów T CD4⁺ we krwi obwodowej w zależności od aktywności klinicznej długotrwałego RZS.

PACjENCI I METODY Do badania włączone 60 pacjentów z RZS i 19 zdrowych ochotników. Pomiarów aktywności choroby, proporcji i liczby różnych subpopulacji limfocytów krwi obwodowej (T, B, komórki NK [natural killer] i NKT [natural killer T cell]), ekspresji CD28 oraz statusu aktywacji limfocytów CD4⁺ dokonano tego samego dnia. Do oceny subpopulacji limfocytów wykorzystano wielokolorową technikę cytometrii przepływowej, z użyciem znakowanych przeciwiek monoklonalnych.

WYNIKI Odsetki limfocytów CD3⁺CD4⁺, NKT, CD4⁺CD28−, CD8⁺CD28−, CD4⁺CD69⁺, CD4⁺CD25⁺ i CD4⁺HLA-DR⁺ były istotnie statystycznie większe u pacjentów z RZS w porównaniu z grupą kontrolną. Wiekowy odsetek komórek CD4⁺CD28⁻ wiązał się z wyższą aktywnością choroby, natomiast odwrotną korelacją zaobserwowano dla limfocytów B. Odsetek komórek CD8⁺CD28⁻ nie był zależny od aktywności choroby. Liczba limfocytów CD4⁺CD69⁺ u pacjentów z RZS zwiększała się wraz z rosnącą wartością DAS28; nie zaobserwowano podobnej zależności dla limfocytów CD4⁺HLA-DR⁺.

WNIOSKI W naszej pracy po raz pierwszy wykazano związek między zmianami fenotypowymi limfocytów krwi obwodowej T, B i NKT a aktywnością RZS u pacjentów z długotrwałą chorobą, co wzmacnia hipotezę o ważnej roli limfocytów krwi obwodowej w modyfikowaniu lub podtrzymywaniu aktywności tej choroby.