Different pattern of T-cell subpopulations in peripheral blood of patients with rheumatoid arthritis at various stages of disease development

Justyna Pawłowska¹, Żaneta Smoleńska², Jacek M. Witkowski¹, Ewa Bryl³

¹ Department of Pathophysiology, Medical University of Gdańsk, Gdańsk, Poland
² Department of Internal Medicine, Connective Tissue Diseases and Geriatrics, Medical University of Gdańsk, Gdańsk, Poland
³ Department of Pathology and Experimental Rheumatology, Medical University of Gdańsk, Gdańsk, Poland

ABSTRACT

INTRODUCTION The comparison of changes in peripheral T-cell subpopulations at different stages of rheumatoid arthritis (RA) development may be important to understand the pathomechanism and to elucidate the course of RA. So far, there have been no comprehensive studies regarding the proportions of T cells in early and long-lasting RA.

OBJECTIVES The aim of this study was to assess the proportion of the main peripheral T-cell subpopulations in patients at various stages of RA development.

PATIENTS AND METHODS We enrolled 75 patients who were divided into 4 subgroups depending on the diagnosis: undifferentiated arthritis (UA), which later developed into RA (UA-RA) and other diseases (UA-non-RA); clinically confirmed untreated RA; long-term treated RA; and the control group. Flow cytometry was used to assess T-cell subpopulations.

RESULTS Patients with clinically confirmed untreated RA differed (P <0.05) in the proportion of CD4⁺ T-cell subpopulations expressing activation markers compared with controls (CD69, CD25, HLA-DR, CD95) and UA patients (CD95). Untreated RA patients had the highest proportion of regulatory CD4⁺ T cells compared with control and other groups. The percentage of CD28⁻ T cells was higher only in the group with clinically confirmed RA but not in those with early RA (at the UA stage).

CONCLUSIONS The peripheral T lymphocyte phenotype in very early RA is not similar to that observed in clinically-confirmed RA. Patients with a confirmed diagnosis of RA can be easily differentiated based on the absolute numbers of the main T-cell subpopulations; however, the percentage of the main T-cell subpopulations do not discriminate those patients in the UA cohort who will develop RA.

KEY WORDS activation marker, CD28, rheumatoid arthritis, T-cell subpopulations, undifferentiated arthritis

INTRODUCTION Rheumatoid arthritis (RA) is a chronic inflammatory disorder, characterized by joint inflammation, progressive joint destruction, and increasing disability. Peripheral immune-mediated reactions are crucial in the pathomechanism of RA both in systemic and local manifestations of the disease.¹ A link between chronic persistent inflammation and oxidative stress participating in the pathogenesis of extra-articular manifestations in RA was also indicated.²

A large effort in research in the last decade has been focused on early RA with the potential for targeting treatment prior to the development of irreversible disability.¹ Early arthritis is frequently undifferentiated at presentation. In line with this, Dixon et al.¹ emphasized that “undifferentiated arthritis” (UA) is a more precise label than “early RA”. On the contrary, established RA is a well-recognized condition and mostly a long-lasting disease.
The course of RA and other forms of rheumatic diseases has been divided into several phases, starting from the preclinical phase leading to the onset of arthritis, followed by the phase of evolution into a specific form of arthritis, and to the final phase of the established arthritis.

The first symptoms of RA begin insidiously and occur episodically. There is a preclinical gap in the course of the disease process between episodes of the first manifestation of arthritis before patients develop persistent synovitis that can clearly be diagnosed as RA.

The preclinical stage of RA and explanation how not-clinically-distinguished arthritis evolves into a typical full-blown RA is the most interesting issue both for clinicians and scientists, from practical and scientific points of view. There are still many unanswered questions regarding the early phases of RA.

The etiology of RA remains to be elucidated. However, the immune mechanism and the role of T cells are of crucial importance. The comparison of the activation states of T cells in different stages of the disease may be important to understand the pathomechanism underlying RA and to elucidate the course of the disease.

It is considered that joint destruction in the course of RA initiated by inflammatory process in the early phase is autonomous in the late phase of the disease. It was shown that the inflammatory features of the synovium are similar in early and long-standing RA.

Considering the facts that the inflammatory features of the synovium have not been shown to change with disease duration and that RA is a systemic disease, a fundamental question arises of whether there are any differences in immunological features in the periphery between early and established RA, with respect to the proportion of the main T-cell subpopulations, which play a fundamental role in the pathomechanism of RA. In our previous work, we demonstrated that RA duration does not affect the proportion of CD4+ T cells with activation markers in patients with established RA with a duration of at least 1 year. To further confirm the involvement of peripheral T cells in the disease course, we showed that the changes in the proportion of activated CD4+ T cells are associated with different disease activities in established long-lasting RA.

The current study was designed to investigate whether there are any differences in the proportions of the main T-cell subpopulations during the course from UA to clinically diagnosed but therapy-naive RA, including the comparison between UA-RA and UA-non-RA patients, and to an established treated long-lasting RA. An additional objective was to investigate whether the proportions of the main T-cell subpopulations could play a role in differentiating early RA from other rheumatic diseases developed among the UA cohort.

We focused on the comparison of the proportions of peripheral CD4+ T cells determined based on the major activation markers and proportions of CD4+ and CD8+ T cells without CD28.

Table 1: Characteristics of the patient subgroups at different stages of rheumatoid arthritis

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Criteria at the time of assessment</th>
<th>Age, y (median, IQR 25%–75%)</th>
<th>Disease duration, mo (median, min–max)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UA-RA</td>
<td>not clinically distinguished peripheral arthritis, disease duration &lt;1 year follow-up of 1–2 years: RA diagnosis</td>
<td>54 (46–57)</td>
<td>5 (3–10)¹</td>
</tr>
<tr>
<td>UA-non-RA</td>
<td>not clinically distinguished peripheral arthritis, disease duration &lt;1 year follow-up of 1–2 years: non-RA diagnosis</td>
<td>44.5 (35–54)</td>
<td>6 (3–10)¹</td>
</tr>
<tr>
<td>diagnosed RA (therapy-naive)</td>
<td>patients fulfilling the 1987 ACR criteria for RA, prior to therapy</td>
<td>54 (27–57)</td>
<td>36 (12–60)</td>
</tr>
<tr>
<td>established RA (during treatment)</td>
<td>patients previously diagnosed as RA, stable disease outcome but still active, no indication to change treatment during a 6-month therapy disease duration &gt;2 years</td>
<td>50 (30–65)</td>
<td>39 (24–84)</td>
</tr>
</tbody>
</table>

Abbreviations: ACR – American College of Rheumatology, IQR – interquartile range, M – men, RA – rheumatoid arthritis, UA – undifferentiated arthritis, UA-RA – UA patients who developed RA, UA-non-RA – UA patients who developed rheumatic diseases other than RA, W – women

¹ statistically significant differences (P < 0.05) between UA-RA vs. RA (diagnosed RA, established RA)
² statistically significant differences (P < 0.05) between UA-non-RA vs. RA (diagnosed RA, established RA)
the classification criteria for any specific rheumatic diseases at the time of enrollment). The median duration of their symptoms was 5 months. Patients were followed from 1 to 2 years; during the follow-up study, standard diagnostic procedures were performed to make the final diagnosis. Phenotyping of T cells was done at the UA stage. Based on the final diagnosis, patients were divided into 2 subgroups as described below:

1. patients who fulfilled the American College of Rheumatology (ACR) criteria for RA were classified as UA-RA after follow-up (8 patients positive for RF and anti-CCP);
2. patients who developed other rheumatic diseases (psoriatic arthritis spondyloarthropathies with peripheral joint involvement, mainly spondylarthritis positive for HLA-B27 antigen, primary Sjögren syndrome) were classified as UA-non-RA after follow-up.

The third subgroup of patients consisted of RA patients who, at the time of enrollment, fulfilled the ACR criteria for RA with a symptom duration between 1 and 5 years (10 positive for RF, 8 positive for anti-CCP). None of those patients used disease-modifying antirheumatic drugs (DMARDs). The subgroup was defined as “diagnosed RA”.

The fourth subgroup, defined as “established RA”, consisted of patients with long-lasting RA (11 positive for RF and anti-CCP) with a symptom duration of longer than 2 years, who, at the time of enrollment, had been on stable DMARD therapy during the last 6 months (methotrexate, 15 mg/wk; glucocorticosteroids, 7.5 mg/d) according to one of the therapeutic principles of the European League Against Rheumatism recommendations described by Bijlsma. Patients did not differ with regard to a treatment protocol and had a comparable time of treatment. During the treatment, the disease activity had been stable for 6 months or longer, but patients did not achieve complete remission defined by the Disease Activity Score 28 (DAS28) as less than 2.6.

Disease activity was measured by DAS28 based on the number of swollen and tender peripheral joints, patients’ overall assessment by the visual analogue scale (VAS), and erythrocyte sedimentation rate (ESR).

For the UA group, the assessment was performed before any DMARDs or steroids were introduced. Additionally, 24 hours before the clinical assessment, patients had not received any non-steroidal anti-inflammatory drugs or paracetamol.

The disease activity was calculated on the same day as peripheral blood collection. The final diagnosis of RA in each group was established according to the 1987 ACR criteria, which allowed us to compare different RA groups.

The control group consisted of 20 age- and sex-matched subjects with no symptoms of joint inflammation and with an ESR and blood morphology within the normal range, no chronic inflammatory or autoimmune diseases, and no cancer in personal or family history. Controls did not have current infections or allergy symptoms at the time of study.

The local ethics committee approved the study and the informed consent was obtained from all patients and controls.

### Flow cytometry of T-cell subpopulations

Flow cytometry was performed to assess the proportions of T-cell subpopulations expressing activation markers, including CD69, CD25, CD95, HLA-DR on CD3+CD4+ T cells and CD28 molecule on CD3+CD4+ and CD3+CD8+ T cells. The gating strategy was performed as described in our previous study.

We carefully identified CD3+CD4+CD25+ cells as regulatory (Treg) features as previously demonstrated.

---

**TABLE 2** Comparison of the clinical assessment between patient subgroups

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Painful joint count (median, min–max)</th>
<th>Swollen joint count (median, min–max)</th>
<th>Patients’ global assessment (VAS), mm (median, min–max)</th>
<th>ESR (median, IQR 25%–75%)</th>
<th>DAS28 (median, IQR 25%–75%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UA-RA</td>
<td>7 (5–11)</td>
<td>3.5 (1–6)</td>
<td>6.5+ (6–7)</td>
<td>25 (22–48)</td>
<td>5.31+ (4.30–5.97)</td>
</tr>
<tr>
<td>UA-non-RA (therapy-naive)</td>
<td>6 (3–10)</td>
<td>2 (1–5)</td>
<td>5 (4–6)</td>
<td>22 (11–35)</td>
<td>4.54 (3.38–5.33)</td>
</tr>
<tr>
<td>diagnosed RA (during treatment)</td>
<td>4 (3–10)</td>
<td>2 (1–6)</td>
<td>5 (4–6)</td>
<td>20 (18–40)</td>
<td>4.10 (3.48–4.88)</td>
</tr>
<tr>
<td>established RA (during treatment)</td>
<td>7 (6–10)</td>
<td>3.5 (2–4)</td>
<td>4.5 (4–7)</td>
<td>25 (16–41)</td>
<td>4.65 (3.80–5.47)</td>
</tr>
</tbody>
</table>

a 0.10 > P > 0.05 in the VAS value between UA-RA and UA-non-RA and diagnosed RA groups

b 0.10 > P > 0.05 in DAS28 between UA-RA and UA-non-RA and diagnosed RA groups

Abbreviations: DAS28 – Disease Activity Score 28, ESR – erythrocyte sedimentation rate, IQR – interquartile range, VAS – visual analogue scale, others – see TABLE 1

ORIGINAL ARTICLE Different pattern of T-cell subpopulations in peripheral blood... 45
Comparison of the proportions of T-cell subpopulations depending on activation markers between the study subgroups: 1 – UA-RA, 2 – UA-non-RA, 3 – diagnosed RA, 4 – established RA, 5 – controls; A – CD4+CD69+, B – CD4+CD25+ activated, C – CD4+HLA-DR+, D – CD4+CD95+; percentages of the T-cell subpopulations were calculated for CD3+CD4+ T cells

Abbreviations: see Table 1

Statistical analysis The significances of differences between quantitative variables were calculated by the Mann–Whitney test, while of those observed between qualitative variables by test of difference between the 2 structure factors using the Statsoft Statistica data analysis software.

All results are presented in tables as medians with 25th and 75th quartiles (interquartile range [IQR]), while graphs (box-and-whisker plots) show the median values, with the 25th and 75th quartiles (IQR) and the minimum and maximum values in whiskers. A $P$ value of less than 0.05 was considered significant.

RESULTS Comparison of the clinical assessment between patient subgroups The baseline clinical characteristics of the patients are summarized in Table 2.

The disease activities measured by overall DAS28 and each component of the factor were comparable between the subgroups.

Patients with UA who developed RA tended to have higher disease activity; however, among each component of DAS28, only the activity of the disease assessed by patients (VAS) differed between the subgroups, while the clinical evaluation components were comparable.
The diagnosis of early RA is currently considered crucial to achieve good therapy outcomes and reversion of the disease, as well as to prevent joint damage and disability. On the other hand, considering the changeable cut-off value for assessing the duration of symptoms, the definition of “early RA” is imprecise. In several original studies, “early RA” was defined as definite RA with disease duration of less than 1 or 2 years. In addition, it should be highlighted that in most of those studies, patients at the time of enrollment fulfilled the criteria for RA, which is why the pathomechanism of RA was not studied at a very early stage of the disease (the UA stage), but at a clinically well-recognized stage. The available evidence indicates that the term “undifferentiated arthritis” is applied to arthritis at the early stage when it cannot be classified into any of the well-defined clinical categories of inflammatory rheumatic diseases. At the UA stage,

The proportion of CD4lowCD25high T-cell subpopulation compared with the control group.

Of interest, only the group with established RA undergoing treatment, but not patients at an earlier stage of the disease, diagnosed RA (therapy-naive), or UA-RA, showed an increased percentage of CD4+CD28– T cells. Additionally, there was a trend towards an increase in the proportion of CD4+CD28– T cells with disease duration in the whole RA group (data not shown). Regarding the proportion of CD8+CD28– T cells, such a difference was observed in patients who met the criteria for RA (diagnosed RA and established RA) regardless of the treatment (FIGURE 3).

**DISCUSSION** RA leads to functional disability, joint damage, and extra-articular disease manifestation, which together result in reduced life expectancy. Joint damage and bone erosions in RA occur early in the disease process, even within the first 3 years. The diagnosis of early RA is currently considered crucial to achieve good therapy outcomes and reversion of the disease, as well as to prevent joint damage and disability.

On the other hand, considering the changeable cut-off value for assessing the duration of symptoms, the definition of “early RA” is imprecise. In several original studies, “early RA” was defined as definite RA with disease duration of less than 1 or 2 years. In addition, it should be highlighted that in most of those studies, patients at the time of enrollment fulfilled the criteria for RA, which is why the pathomechanism of RA was not studied at a very early stage of the disease (the UA stage), but at a clinically well-recognized stage.

The available evidence indicates that the term “undifferentiated arthritis” is applied to arthritis at the early stage when it cannot be classified into any of the well-defined clinical categories of inflammatory rheumatic diseases. At the UA stage,
the identification of the subset of patients who will develop RA is a challenge both for clinicians and researchers. Accordingly, the pathomechanism of early RA should be studied at the UA stage. In line with the above, UA patients included in our study did not fulfill any of the existing classification criteria for any specific rheumatic disease at the time of enrollment. Patients with a definitive diagnosis at baseline and with a documented duration of symptoms of more than 1 year were categorized as diagnosed RA (therapy-naive) or established RA (during treatment).

The limitation of the present study is a relatively small number of patients; nevertheless, this could mean that the differences in some variables are smaller than they really are. To limit the heterogeneity of treated RA patients, we performed a careful patient selection. The treated RA subgroup included only patients receiving a fixed dose of methotrexate and glucocorticosteroid without any other drugs known to directly affect lymphocytes. All diagnosed RA patients were positive for RF and anti-CCP. The design of the UA cohort did not allow to select patients because the immunological analysis was performed before diagnostic tests; however, when analysed retrospectively, 80% of the UA-RA patients were positive for RF and anti-CCP, while only 50% and 40% of the UA-non-RA patients were positive for RF and anti-CCP, respectively. Of note, among 54 patients with UA only 10 were diagnosed with RA (even with the new 2011 ACR/EULAR criteria), which makes it challenging to study this group of patients.

Several studies showed similar features of the synovium in early RA and later stages of the disease. The comparison of inflammatory features in the synovium between patients with early RA, with disease duration of less than 1 year, with long-lasting RA (1–5 years), and with disease duration of longer than 5 years showed that the changes are not dependent on disease duration. The hyporesponsive state of T cells infiltrating the synovium in long-lasting RA (>5 years) is similar to that in early RA (<1 year). The observation that the expression of matrix metalloproteinases and tissue inhibitor of metalloproteinases in the synovium did not differ between early (<1 year) and long-lasting RA (>5 years). It seems that the synovial inflammation at the tissue molecular level in early RA is basically the same as that observed in late RA.

To the best of our knowledge, the numerical changes of peripheral T-cell subpopulations determined in peripheral blood in the early phase of RA (the UA stage) – in the context of established RA – have not been extensively studied so far. Several studies showed similar features of the synovium in early RA and later stages of the disease. The comparison of inflammatory features in the synovium between patients with early RA, with disease duration of less than 1 year, with long-lasting RA (1–5 years), and with disease duration of longer than 5 years showed that the changes are not dependent on disease duration.

Smeets et al. demonstrated that the hyporesponsive state of T cells infiltrating the synovium in long-lasting RA (>5 years) is similar to that in early RA (<1 year). Katrib et al. indicated that the expression of matrix metalloproteinases and tissue inhibitor of metalloproteinases in the synovium did not differ between early (<1 year) and long-lasting RA (>5 years). It seems that the synovial inflammation at the tissue molecular level in early RA is basically the same as that observed in late RA.

Considering this finding, our study suggests that activated T cells can inhibit the function of Treg cells in early and later stages of RA but before treatment. This explains why, despite the high number of peripheral Treg cells, patients had high disease activity.

Another possible explanation could be an impaired function of Treg cells in RA patients. Ehrenstein et al. demonstrated an impaired function of CD4+CD25+ T cells isolated from the blood of patients with active RA during DMARD treatment. A remarkable CD4+CD28− subpopulation has been widely studied in autoimmune diseases, indicating its role in tissue damage. An increased proportion of both CD4+CD28− and CD8+CD28− T-cell subpopulations in the peripheral blood of established RA patients compared with the control group was demonstrated. This result can also be linked to the process of premature aging of T cells observed in RA. In addition, the presence of increased CD4+CD28− T-cell subpopulation was associated with a high level of tumor necrosis factor α (TNF-α). It was further found that treatment with anti-TNF-α leads to an increase in the number of CD28 molecules on the surface of CD4+ T cells. The results suggest that the loss of CD28 antigen on the surface of lymphocytes results from severe chronic inflammation. These
findings raise the question about the contribution of CD28- T cells to the local inflammatory process involving joints. A number of studies did not show any increase in the proportion of these subpopulations in the synovial fluid in RA and any correlation with the disease activity.27 However, in our previous study, we showed a significant contribution of CD28 cells in the synovial membrane of RA.28

In agreement with the results, we showed an increase in the percentage of CD4+CD28- in treated patients with long-lasting RA. An increase in the proportion of CD8+CD28- was indicated both in the diagnosed and established groups of RA regardless of treatment. Interestingly, we did not show a higher percentage of the CD8- subpopulation in early RA (the UA stage).

We showed discrepancies in the proportion of the main T-cell subpopulations between patients at different clinical stages of RA. Patients with confirmed RA (clinically diagnosed RA) show numerous differences in the proportion of T-cell subpopulations compared with controls and other patient subgroups and, therefore, can be easily distinguished based on the phenotype status of the peripheral T cells, while the percentages of the main T-cell subpopulations do not discriminate patients who will develop RA at an undifferentiated stage of the disease process from the UA cohort. We have recently shown that the proliferation status of CD4+ T cells may distinguish early RA in the UA cohort.24 Based on the results, it seems that the proliferation kinetics works much better as a discrimination factor than T-cell phenotyping at the UA stage.

Regarding numerical changes in peripheral T cells, our results suggest that some changes can be only observed in confirmed RA but not in the early undifferentiated disease. Based on the numerical changes in T cells, the results suggest that the immune process related to peripheral T cells in early RA (the UA stage) is not similar to that observed in clinically distinguished RA.

Acknowledgements This work was supported by the Medical University of Gdańsk’s intramural grants (ST-58 [granted to J.M.W.], W-112 and MN-112 [granted to J.P.]) and the Polish National Science Centre’s grant (N N402 640 440; granted to E.B.).

REFERENCES


ARTYKUŁ ORYGINALNY

Odzienna dystrybucja subpopulacji limfocytów T we krwi obwodowej pacjentów z reumatoidalnym zapaleniem stawów w różnych stadiach rozwoju choroby

Justyna Pawłowska¹, Żaneta Smoleńska², Jacek M. Witkowski¹, Ewa Bryl³

¹ Katedra i Zakład Fizjopatologii, Gdańskii Uniwersytet Medyczny, Gdańsk
² Katedra i Klinika Chorób Wewnętrznych, Chorób Tkanki Łącznej i Geniatrii, Gdańskii Uniwersytet Medyczny, Gdańsk
³ Zakład Patologii i Reumatologii Doświadczalnej, Gdańskii Uniwersytet Medyczny, Gdańsk

SŁOWA KLUCZOWE
CD28, marker aktywacji, nieróżnicoowiane zapalenie stawów, reumatoidalne zapalenie stawów, subpopulacje limfocytów T

STRESZCZENIE

WPROWADZENIE
Porównanie zmian dotyczących subpopulacji limfocytów T krwi obwodowej w różnych stadiach zaawansowania reumatoidalnego zapalenia stawów (RZS) może być istotne w zrozumieniu patomechanizmu i wyjaśnieniu przebiegu RZS. Dotychczas nie przeprowadzono kompleksowych badań dotyczących różnic w odsetkach limfocytów T zarówno we wczesnym, jak i w długotrwałym RZS.

CELE
Celem tego badania była ocena odsetka głównych subpopulacji limfocytów T krwi obwodowej u pacjentów w różnych stadiach rozwoju RZS.

PACJENCI I METODY
Do badania włączono 75 pacjentów, których podzielono na 4 podgrupy w zależności od rozpoznania: nieróżnicoowane zapalenie stawów (undifferentiated arthritis – UA), które później rozwinęło się w RZS (UA-RZS) i inne choroby (UA-nie-RZS), ustalone klinicznie nieleczone RZS, długotrwałe RZS w trakcie leczenia, oraz grupę kontrolną. Do oceny subpopulacji limfocytów T zastosowano cytometrię przepływową.

WYNIKI
U nieleczeni o pacjentów z klinicznie ustalonym rozpoznaniem RZS wykazano różnice (p <0,05) w odsetku subpopulacji limfocytów CD4+ z ekspresją markerów aktywacji w porównaniu z grupą kontrolną (CD69, CD25, HLA-DR, CD95) i z grupą UA (CD95). Nieleczeni pacjenci z RZS mieli największy odsetek limfocytów T CD4+ regulatorowych w porównaniu z grupą kontrolną i innymi podgrupami pacjentów. Odsetek limfocytów T CD28 był większy tylko w grupie z ustalonym rozpoznaniem RZS, ale nie z wczesnym RZS (w stadium UA).

WNIOSKI
Fenotyp limfocytów T krwi obwodowej w bardzo wczesnym RZS nie jest podobny do obserwowanego w klinicznie rozpoznanym RZS. Pacjentów z ustalonym rozpoznaniem RZS można łatwo różnicować na podstawie bezwzględnej liczby głównych subpopulacji limfocytów T, jednak procent głównych subpopulacji limfocytów T nie ułatwia różnicowania w kohorcie UA pacjentów, u których rozwinię się RZS.