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Original Article

Changes in peripheral blood T lymphocyte subsets predict disease progression in patients with rheumatoid arthritis

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Abstract: Objective: To explore the correlation between abnormal changes in peripheral blood T lymphocyte subsets and disease progression in patients with active rheumatoid arthritis (RA). Methods: This is a retrospective study, in which 53 patients with active RA were selected as the study subjects and 50 healthy people were selected as the control group. Lymphocyte subsets were determined in both arms. According to whether CD4/CD8 ratio increased, RA patients were subdivided into an elevated CD4/CD8 group and a non-elevated CD4/CD8 group, and compared to the control group. The risk factors affecting the disease progression of patients with active RA were analyzed. Results: $CD4^+$ T lymphocyte subsets in patients with increased CD4/CD8 were significantly higher than those in healthy controls. In addition, the elevated CD4/CD8 group showed significantly $CD8^+$ T lymphocyte subsets than the non-elevated CD4/CD8 group and the control group ($P<0.05$). The $CD4^+$ T lymphocyte subsets in the elevated CD4/CD8 group were not significantly higher than those in the non-elevated CD4/CD8 group ($P>0.05$). The $CD3^+$ T lymphocyte subsets as well as $CD19^+$ B and NK lymphocyte subsets showed no significant difference among the three arms ($P>0.05$). In addition, CD4, CD8 and CD4/CD8 were identified to be the risk factors affecting disease progression in patients with active RA. Conclusions: When an autoimmune disorder occurs in patients with active RA, $CD8^+$ T lymphocyte subsets are significantly suppressed, while $CD4^+$ T lymphocyte subsets show different manifestations, with some patients presenting no obvious increase. In addition, CD4, CD8 and CD4/CD8 can help to indicate the risk of disease progression in patients with active RA.

Keywords: Rheumatoid arthritis, T lymphocyte subsets, difference analysis, nosogenesis

Introduction

Rheumatoid Arthritis (RA) is a systemic autoimmune disease characterized by chronic and progressive multiarticular synovitis and destruction of articular cartilage and bone, which can recur and lead to joint dysfunction, with a disability rate of up to 15% [1]. It accounts for about 1% of the global population and is the most common systemic autoimmune disease in the world. RA has a predilection for middle-aged women, and the risk increases with age [2]. Its clinical manifestations are symmetrical multi-joint swelling and pain in the hands, wrists, feet and other parts [3]. Although its treatment strategy has been continuously optimized, the therapeutic effect of RA is still not ideal due to the complex pathologic mechanism and long course of disease [4]. Therefore, studying the pathologic mechanism of RA is of great significance to further optimize the treatment of RA and reduce the disability rate of patients.

Clinical research has shown [5], that the pathogenesis of RA is closely associated with patients' autoimmune impairment, while abnormal lymphocyte function and apoptosis disorder are predisposing factors for autoimmune diseases. The imbalance of immune cells was shown to play an important role in the pathogenesis of RA [6, 7]. However, no consensus has developed concerning the relationship between abnormal T lymphocyte subsets and RA. Some studies suggest that RA is related to T

lymphocyte abnormalities, but some studies believe that the two are irrelevant [8, 9]. Accordingly, this study mainly explores the differences of clinical indicators in RA patients with different CD4/CD8 ratios to further understand the association between them, so as to provide reference for clinical diagnosis and treatment of RA. The novelty of this study lies in the analysis of peripheral blood lymphocyte subtypes with or without elevated CD4/CD8 ratio and the comparison with other clinical indicators. Furthermore, we analyzed the correlation between peripheral blood lymphocyte subtypes and patient disease progression, as well as risk factors influencing disease progression in patients with active RA.

Participants and methods

Research participants

This was a retrospective study. Fifty-three patients with RA admitted to the inpatient department of Hubei Provincial Hospital of TCM from April 2020 to November 2020 were selected as research participants. All the patients were selected according to the 2010 American College of Rheumatology (ACR)/European League against Rheumatism (EULAR) classification criteria [10], without serious primary diseases such as cardiovascular, hepatorenal and hematopoietic diseases or other autoimmune diseases. There were 15 males and 38 females aged from 30 to 74 years old (average: 48.20±10.62) with a course of disease ranging from 1 to 19 years. The Ethics Committee at the Hubei Provincial Hospital of TCM approved this study without reservation, and each patient signed the informed consent prior to enrollment. Further, patients were subdivided into elevated CD4/CD8 group and non-elevated CD4/CD8 group based on the increase of CD4/CD8 ratio. Additionally, 50 healthy controls, including 20 males and 30 females aged from 24 to 65 years old (mean: 46.84±9.31), who concurrently underwent physical examination in our hospital were selected as the control group.

Research methods

Specimen preparation: Fasting venous blood (6 mL) was collected from patients into EDTA-K2 anticoagulation tubes, sodium citrate coagulation tubes and separate gel coagulation-promoting tubes respectively for later use. The samples were determined within 6 h after collection.

Routine index detection: The absolute value of lymphocytes was determined by DXH 800 Hematology Analyzer (Beckman Coulter); Erythrocyte sedimentation rate (ERS) was measured by SD-100 dynamic hematocrit tester (Beijing Succeeder Technology Inc.); rheumatoid factor (RF) was detected by AU5821 automatic biochemical analyzer (Beckman Coulter).

Detection of peripheral blood lymphocyte subsets by flow cytometry: FACSCalibur flow cytometry (Becton Dickinson, USA) was used to detect peripheral blood lymphocyte subsets. Specifically, 100 μL of venous whole blood from the EDTA-K2 anticoagulation tubes was put into the loading tube of the flow cytometer, and added with 20 μL of CD4FITC/CD8PE/CD3PC5 antibody (Hengfei Biological Technology Co., Ltd., Shanghai, China, A07750+A07757+A07749), CD3FITC/CD19PE antibody (SeeBio Biotech Co., Ltd., Shanghai, China, DC005) and CD3FITC/CD16CD56PE fluorescently labeled monoclonal antibody (Hengfei Biological Technology Co., Ltd., Shanghai, China, 319101), respectively, for 40 min of light-tight cultivation. Then, 2 mL hemolysin was added to lyse the red blood cells, followed by two phosphate buffer solution (PBS) rinses and sample loading for analysis. The acquisition of cell information employed Cell Quest Pro acquisition software. For each sample 10,000 viable lymphocytes were gated, following size (forward scatter, FSC) and granularity (side scatter, SSC) parameters. The experimental results were processed by Cell Quest Pro analysis software (**Figure 1**).

Clinical efficacy evaluation and laboratory examination

Gender, age, course of disease, the Disease Activity Score in 28 joints (DAS28) [11], absolute value of lymphocytes, and rheumatoid factor (RF) were compared between the two groups.

Criteria for determining active RA: (1) Morning stiffness time ≥15 min; (2) Feeling of weakness within 6 hours after getting up in the morning; (3) Movement-related pain or tenderness in

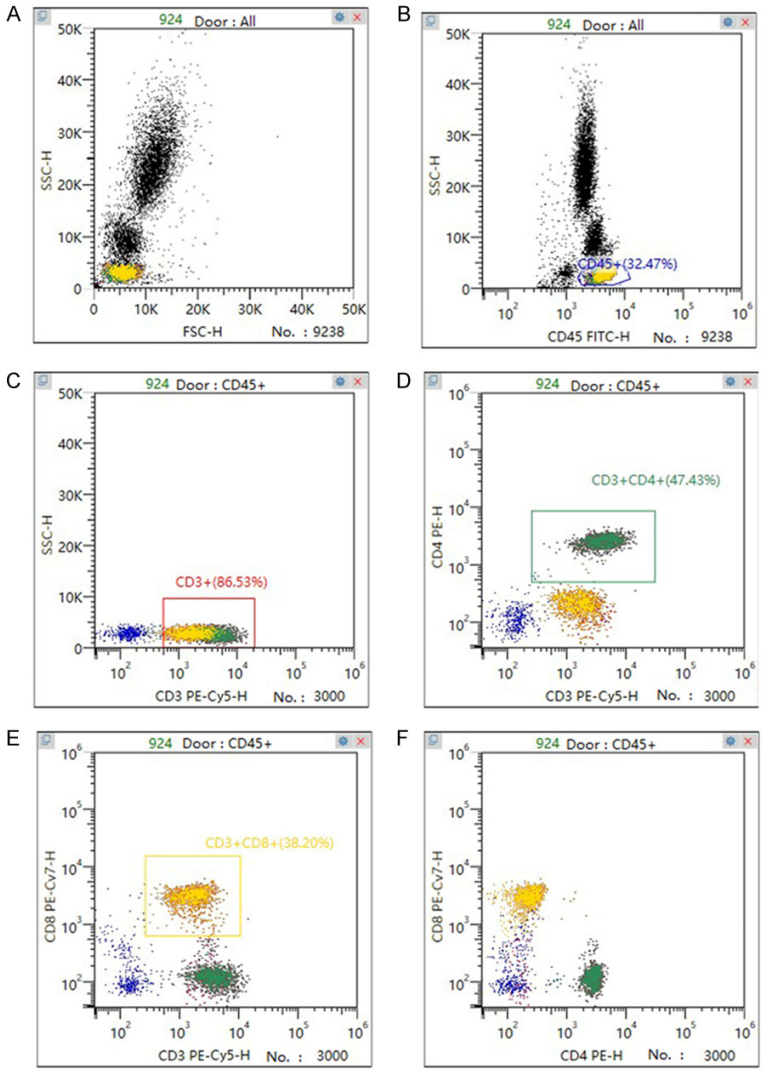

Figure 1. Flow cytometry analysis of peripheral blood lymphocyte subsets. A. Lymphocyte nucleus. B. Lymphocytes. C. Gating lymphocytes. D. T helper cells. E. T suppressor cells. F. CD4 and CD8 distribution map.

more than 2 peripheral joints; (4) Synovium swelling in more than 2 peripheral joints; (5) ESR exceeding 20 mm/h and 30 mm/h in male and female respectively.

Calculation of DAS28: The DAS28 ranges from 0 to 10 points, and higher scores indicate higher disease activity. The specific methods are as follows: (1) The number of tender joints: the number among 28 joints (T28) of bilateral proximal interphalangeal joint, metacarpophalangeal joint, wrist joint, elbow joint, shoulder joint and knee joint with tenderness was counted; (2) The number of swollen joints: the number of swollen joints (SW28) of the above 28 joints was checked; (3) DAS28 was calculated according to the following formula in combination with ESR:

DAS28 = [0.56 × sqrt (T28) + 0.28 × sqrt (SW28) + 0.70 × Ln (ESR)] × 1.08 + 0.16

The disease activity of RA patients was divided into either remission stage (DAS28< 2.6) or active stage based on their DAS28 scores. The active stage can be further subdivided into three groups: low activity group (2.6< DAS28<3.2), moderate activity group (3.2<DAS28<5.1) and high activity group (DAS-28>5.1) [12].

Statistical methods

SPSS25.0 software was used for data analysis. Measured data conforming to normal distribution were expressed by $\bar{x} \pm s$, and the comparison between groups was performed by the t-test. Counted data were expressed as cases/percentages (n/%), and the Chi-square test was used for comparison of those data between groups. When the theoretical frequency was less than 5 in the Chi-square test, the continuous correction Chi-square test was used. Spearman's correlation coefficient was used to evaluate the correlations of CD4 and CD8 with the development of patients' disease and logistic regression was performed to analyze the risk factors affecting the disease progression of patients with active RA. P<0.05 was the significance level in all analyses.

Results

Baseline data

There were no significant differences in gender, age, mean age, mean course of disease, history of rheumatoid arthritis, degree of education, drinking history, residence and marital

Table 1. Baseline data of patients in the two groups [n (%), mean ± SD]

Variable	n	Patient group (n=53)	Control group (n=50)	χ^2/t	P
Gender				1.569	0.210
Male	35	15 (28.30)	20 (40.00)		
Female	68	38 (71.70)	30 (60.00)		
Age (years old)				0.918	0.338
<50	61	29 (54.72)	32 (64.00)		
≥50	42	24 (45.28)	18 (36.00)		
Average age (years old)	103	48.20±10.62	46.84±9.31	0.689	0.492
Average course of disease (years)	103	7.74±4.40	-		
History of rheumatoid arthritis				2.443	0.118
No	82	39 (73.58)	43 (86.00)		
Yes	21	14 (26.42)	7 (14.00)		
Degree of education				2.202	0.532
Primary school	22	14 (26.42)	8 (16.00)		
Secondary school	36	16 (30.19)	20 (40.00)		
Junior college or undergraduate	26	14 (26.42)	12 (24.00)		
Bachelor degree or above	19	9 (16.98)	10 (20.00)		
History of drinking				0.349	0.555
No	65	32 (60.38)	33 (66.00)		
Yes	38	21 (39.62)	17 (34.00)		
Residence				1.094	0.296
Urban	69	38 (71.70)	31 (62.00)		
Rural	34	15 (28.30)	19 (38.00)		
Marital status				0.001	0.973
Single	29	15 (28.30)	14 (28.00)		
Married	74	38 (71.70)	36 (72.00)		

Table 2. Comparison of absolute value of peripheral blood lymphocyte subtypes ($\times 10^9$/L)

Groups	$CD3^+$	$CD4^+$	$CD8^+$	$CD19^+$	NK	$CD4^+/CD8^+$
Elevated CD4/CD8 group	1.05±0.43	0.76±0.29*	0.25±0.11$^{*,\Delta}$	0.19±0.14	0.20±0.16	3.31±1.36$^{*,\Delta}$
Non-elevated CD4/CD8 group	1.10±0.40	0.62±0.23	0.44±0.21	0.21±0.15	0.25±0.13	1.51±0.48
Control group	0.98±0.38	0.52±0.20	0.41±0.19	0.17±0.07	0.26±0.18	2.0±1.26

Note: *P<0.05 *vs* the control group; $^\Delta$P<0.05 *vs* the non-elevated CD4/CD8 group.

status between the two groups (P>0.05), indicating comparability. See **Table 1**.

Changes of lymphocyte subsets in each group

The CD4/CD8 ratio was significantly higher in the elevated CD4/CD8 group than in the non-elevated CD4/CD8 group and the control group. The $CD4^+$ T lymphocyte subsets in patients with increased CD4/CD8 ratio were significantly higher than those of controls (P<0.05). The $CD8^+$ T lymphocyte subsets were significantly lower in the elevated CD4/CD8 group compared with the other two groups (P<0.05). The $CD4^+$ T lymphocyte subsets in the elevated CD4/CD8 group were not significantly higher than those in the non-elevated CD4/CD8 group (P>0.05). There were no significant differences in $CD3^+$ T lymphocyte subsets, $CD19^+$ B lymphocyte subsets, and NK lymphocyte subsets among the three arms (P>0.05). See **Table 2**.

Comparison of other clinical evaluation indexes

RF and DAS28 scores differed insignificantly between the elevated CD4/CD8 group and non-elevated CD4/CD8 group (P>0.05). There

Table 3. Comparison of other clinical indexes

Group	Absolute value of lymphocytes ($\times 10^9$/L)	Rheumatoid factor (IU/mL)	DAS28
Elevated CD4/CD8 group	1.49±0.51	128.91±153.65	6.15±1.52
Non-elevated CD4/CD8 group	1.58±0.53	166.78±200.77	6.62±1.49
Control group	1.43±0.56	/	/

was no significant difference in the absolute value of lymphocytes among the three arms (P>0.05). See **Table 3**.

Correlations of peripheral blood lymphocyte subtypes with disease progression in patients with RA

The research participants in this study all had moderate or high disease activity, so DAS28 between 3.2 and 5.1 was recorded as moderate disease activity and indicated as 1, while DAS28 larger than 5.1 was recorded as high disease activity and indicated as 2. Pearson correlation coefficient was used to analyze the correlations of the absolute values of peripheral blood lymphocyte CD4, CD8 subtypes and CD4/CD8 ratio with disease progression in patients with RA. The results showed that CD4 and CD4/CD8 were positively correlated with disease progression (r=0.424, P<0.01; r=0.398, P<0.01), while CD8 was negatively correlated with it (r=-0.405, P<0.01). See **Figure 2**.

Analysis of risk factors affecting disease progression in patients with active RA

The factors with differences (CD4, CD8 and CD4/CD8) were included and assigned as independent variables, and the progression of active RA (with or without) was used as the dependent variable, for multivariate analysis using the multivariate logistic regression model. The results identified that CD4 (P=0.043), CD8 (P=0.026) and CD4/CD8 (P=0.004) were the factors affecting the disease progression of patients with active RA. See **Tables 4** and **5**.

Discussion

RA is a chronic autoimmune disease with synovitis as the main clinical feature, and is internationally recognized as a major refractory disease [13]. Clinically, the pathogenesis of the disease remains to be defined, but it is believed to be the result of a combination of factors such as heredity, environment, sex hormones, and infection [14, 15]. Clinical studies suggest that the disorder of cellular immune and the imbalance of cell subsets are closely related to the occurrence and development of RA. In the past, lymphocyte subgroups were classified in peripheral blood samples of patients and cells related to autoimmunity (mainly including T cells, B cells and NK cells) were observed to determine whether patients have cellular immune disorders.

In this study, we found no significant difference in $CD19^+$ B lymphocyte subsets and NK lymphocyte subsets among the three arms, suggesting that the two subsets play a minor role in the pathogenesis of RA, which is consistent with the conclusions of other experiments [16, 17]. $CD3^+$ is a common marker antigen expressed by T lymphocytes, which can be further divided into two main subsets: $CD4^+$ cells and $CD8^+$ cells [18]. The experimental data showed that the total absolute value of $CD3^+$ lymphocytes had no obvious difference between cases and controls, but the absolute values of other two subtypes (CD4 and CD8) differed significantly. Among them, $CD4^+$ cells are helper or effector T cells, which have the function of transforming helper T cells into effector cells, facilitating B cells to produce antigens and activating macrophages, so as to assist and induce cellular or humoral immunity [19]. $CD8^+$ cells are suppressor or killer cells, which can inhibit T cell activation or B cell production of antibodies and cytotoxicity to inhibit cellular or humoral immunity [20]. In general, CD4/CD8 increases abnormally when autoimmune diseases are in the active stage. In this study, 53 patients with active RA were evaluated by the DAS28 score, and all of them were found to be of medium and high disease activity. Correlation analysis showed that CD4 and CD4/CD8 were positively correlated with disease progression in patients with active RA, while CD8 was negatively correlated with it. In addition, lymphocyte subsets testing revealed that 33.96% (18/53) patients did not have an increased CD4/CD8 ratio, mainly because $CD4^+$ cells were not significantly increased in these

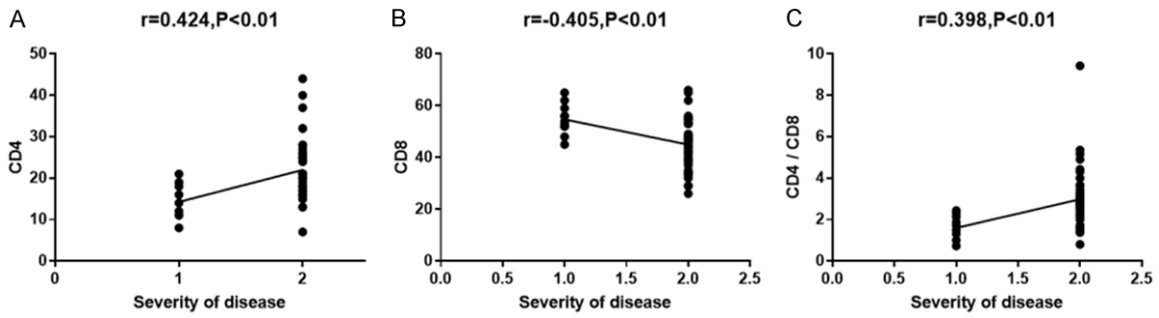

Figure 2. Correlations of peripheral blood lymphocyte subtypes with disease progression in patients with active RA. A. CD4 was positively correlated with the disease progression of RA patients (r=0.424, P<0.01). B. CD8 was negatively correlated with the disease progression of RA patients (r=-0.405, P<0.01). C. CD4/CD8 was positively correlated with the disease progression of RA patients (r=0.398, P<0.01).

Table 4. Assignment of multivariate Logistic regression analysis

Variables	Variables	Assignment
CD4	X1	Continuous variable
CD8	X2	Continuous variable
CD4/CD8	X3	Continuous variable

Table 5. Multivariate analysis of variables affecting disease progression in patients with active rheumatoid arthritis

Factor	β	S.E	Wald	P	OR	95% CI
CD4	1.352	0.690	4.051	0.043	3.952	1.013-15.108
CD8	1.562	0.684	5.162	0.026	4.786	1.283-18.363
CD4/CD8	2.307	0.787	8.640	0.004	10.032	2.175-46.185

patients. Moreover, we found that changes in the absolute value of $CD4^+$ T lymphocyte subsets could not be used as a detection index of cellular immune disorder in active RA patients.

Based on the experimental results, it is concluded that the reasons for such differences may be: (1) The abnormal distribution of T lymphocyte subsets may be related to the different stages of disease development in patients; (2) The influence of T lymphocytes on the pathogenesis of RA may lie in the functional changes of T lymphocytes by changing individual surface specific proteins, leading to autoimmune injury [21]. So it is not a change in quantity that matters, it is a change in function or activity; (3) The lymphocytes that play a decisive role in the mechanism belong to a small number of cell subsets, and the change of their distribution has no obvious influence on the absolute value and proportion of the whole T lymphocyte subsets [22, 23]. Therefore, in our subsequent research on the pathogenesis of RA, patients with active RA whose CD4/CD8 ratio is not increased can be selected to search for a class of targeted cells with higher correlation with RA when the cellular immune function changes abnormally. In addition, the Logistic regression analysis found that CD4, CD8, and CD4/CD8 were independent risk factors affecting the disease progression of patients with active RA, indicating that the three indicators can indicate the exacerbation of RA to a certain extent.

This study confirmed that the changes in peripheral blood T lymphocyte subsets can predict the disease progression of RA patients, and CD4, CD8 and CD4/CD8 are risk factors affecting disease progression, which has clinical significance for evaluating and preventing disease progression in RA patients. However, there is room for improvement in this study. First, we can supplement the basic research on peripheral blood T lymphocyte subsets to further explore the underlying logic of the correlation between peripheral blood T lymphocyte subsets and disease progression in patients with RA. Second, gene-derived molecules that affect peripheral blood T lymphocyte subsets can be searched, which is significant for the regulation of RA progression. We will gradually improve the follow-up research in the future.

To sum up, this study focuses on the correlation between cellular immune dysfunction and the pathogenesis of RA, and finds that the imbalance of immune response will inevitably lead to patient-related pathologic damage. However, at

present, the changes of T lymphocyte subsets such as $CD4^+$ or $CD8^+$ are of limited value for clinical diagnosis and treatment, so we should search for more targeted lymphocyte subsets, and consider focusing the screening range on patients with active RA and non-increased CD4/CD8. On the other hand, $CD4^+$ and $CD8^+$ T lymphocytes can help identify people at high risk of developing RA.

Disclosure of conflict of interest

None.

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