Absolute reduction of peripheral regulatory T cell in patients with relapsing polychondritis

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Abstract

Objective

Although relapsing polychondritis (RP) is considered as an immune-mediated systemic disease, the levels of peripheral lymphocyte subpopulations are rarely studied in patients with RP. In this study, we focused on changes of peripheral CD4+T cell subsets in patients with RP.

Methods

Absolute numbers and percentages of CD4+T cell subsets including helper T(Th)1, Th2, Th17 cells and regulatory T (Treg) cells in peripheral blood (PB) from 19 RP patients, healthy controls and RA patients respectively were assessed by flow cytometry combined a microbead-based single-platform method. We compared the CD4+T cell levels in all RP patients and healthy controls. In addition, we analysed the difference of the absolute number and percentage of Treg cells between RP and RA patients.

Results

Compared with healthy controls, all RP patients had significantly both lower absolute number and proportion of Treg cells (absolute number, 45.10/μl vs. 22.48/μl, p<0.001; proportion, 5.19% vs. 3.78%, p<0.001) no matter whether they had received treatment or not. Similarly, the absolute number of Th2 cells in all RP patients was decreased (10.19/μl vs. 7.44/μl, p=0.030). However, there were no significant differences in percentages and absolute numbers of Th1 and Th17 cells between RP patients and healthy controls. The above results led to increased ratios of Th1/Treg (3.68 vs. 2.06, p=0.020), Th2/Treg (0.29 vs. 0.21, p=0.037) and Th17/Treg (0.25 vs. 0.14, p<0.001) in RP patients, and untreated RP patients were mainly characterised by the imbalance of Th17/Treg (0.25 vs. 0.14, p<0.01). There was no significant difference in Treg cells between RP and RA patients (p>0.05).

Conclusion

Our data suggest that the reduction of Treg cells and its imbalance with Th cells play an important role in the pathogenesis of RP.

Key words

relapsing polychondritis, regulatory T cell, helper T cell

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Received on January 20, 2020; accepted in revised form on May 5, 2020.

Funding: this work was supported by a grant from the National Natural Science Foundation of China (81971543 and 81471618) and the Key Research and Development Project of Shanxi Province in China (201803D31119).

Competing interests: none declared.

Introduction

Relapsing polychondritis (RP) is a rare systemic disease characterised by repeated inflammation and progressive destruction of cartilage and connective tissue, leading to rhinochondritis, auricular chondritis, scleritis, conjunctivitis, uveitis, arthritis and so on (1). Ultimately, the continuous destruction of cartilage tissue causes dysfunction or failure of related tissues or organs.

It is generally believed that the onset of RP is related to the attack of cartilage tissue by abnormal cellular and humoral immune functions. This is supported by the facts that, the early pathological changes of auricle chondritis in RP patients were characterised by perfusion of lymphocytes, macrophages, neutrophils and plasma cells (2) and the lesions were mainly infiltrated by $CD4^+$T cells during the active period of the disease (1). Naïve T cells can differentiate into four different subtypes of $CD4^+$T cells under different cytokines and environmental effects. Among them, Th1($CD4^+IFN\text{-}\gamma^+$), Th2($CD4^+IL\text{-}4^+$), and Th17($CD4^+IL\text{-}17^+$) cells are the major effector T cells and play pro-inflammatory roles in autoimmune diseases, while $CD4^+CD25^+Foxp3^+$ regulatory T (Treg) cells play an anti-inflammatory role and contribute to the immune balance. Previous studies on autoimmune diseases had mostly focused on the overactivity of effector T cells to self-antigens, which was generally considered as the major pathogenesis of immune disorders and the basis of immunosuppressive therapies with an increased risk of infection and tumorigenesis.

Recently, the increasing findings showed that the quantitative and functional deficiencies of Treg cells result in the imbalance with effector T cells in many autoimmune diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) (3-5). However, the changes of these subtypes, especially Treg cells, in RP patients remain unknown. Therefore, studies on peripheral Treg cells of RP patients help to provide a new therapeutic strategy that does not lead to increased incidence of infection and tumour.

In this study, to determine whether the reduction of peripheral Treg cells associates with the pathogenesis of RP, we analysed the levels of $CD4^+$T cell subsets in untreated and treated patients with RP and compared them with those in the healthy controls respectively, as well as with each other. RA patients were also selected as controls to observe the difference of Treg cells between RP and other autoimmune diseases.

Material and methods

Study population

Nineteen RP patients admitted to the Department of Rheumatology at the Second Hospital of Shanxi Medical University between December 2015 and October 2019 were enrolled in the study. These patients were diagnosed as RP according to the criteria provided by Damiani *et al*.: 1) binaural chondritis; 2) non-erosive seronegative polyarthritis; 3) nasal chondritis; 4) ophthalmia, including conjunctivitis, keratitis, scleritis, superficial scleritis and uveitis; 5) laryngeal and/or tracheal chondritis; 6) cochlear and/or vestibular damage. One of the following conditions can be diagnosed: 1) three or more of the above six articles; 2) satisfying the above one plus pathological confirmation, such as ear and nasal respiratory cartilage biopsy; 3) the lesion involved two or more anatomical sites and it was effective in the treatment of steroids or dapsone. Of them, 13 patients had never received any treatment. Control groups included 19 gender-matched and age-matched healthy adults and rheumatoid arthritis (RA) respectively. RA patients fulfilled the 1987 rheumatoid arthritis classification criteria of American College of Rheumatology (ACR). The study was approved by the local ethics committee and written informed consent was obtained from all participants.

Clinical data collection

Medical history and laboratory data from all patients were collected. The medical history includes age, gender, age of symptom onset, clinical symptoms, and medication. Laboratory data includes erythrocyte sedimentation rate (ESR), C-reaction protein (CRP), immunoglobulin (Ig) G, M, A, and autoantibodies, including rheumatoid fac-

tor antigen (RF), anti-nuclear antibody (ANA), anti-neutrophil cytoplasmic antibody (ANCA) and anti ENA antibody (anti-ENA). CRP was detected by turbidimetric inhibition immunoassay and positive values were greater than or equal to 8 mg/L. ESR was measured by the Westergren method, and values >15 mm/h for men and >20 mm/h for women were considered abnormal. Autoantibodies and immunoglobulin were tested by enzyme-linked immunosorbent assay (ELISA).

Flow cytometry

To detect Th1, Th2 and Th17 cells, 10μl of PMA, 10μl of Ionomycin and 1μl of Golgi Stop were added to 80μl of anti-coagulated blood and incubated at 37°C for 5 hours. The samples were divided into A and B tubes and labelled with anti-CD4-FITC at room temperature for 30 min in the dark, using Fixation/Permeabilisation to fix avoid light for 30 minutes at 4°C. The sample in tube A was bound to anti-IL-4-PE, anti-IFN-γ-APC, and the sample in tube B was bound to human anti-IL-17-PE protected from light at room temperature for 30 minutes. The cells were washed with PBS and detected by four-colour flow cytometry. When Treg cells were detected, 80μl of anticoagulant was added to anti-CD4-FITC and anti-CD25-APC and left to stand in the dark at room temperature for 30 minutes. The sample was added with 1ml of fresh Fixation/Permeabilisation for 30 minutes at 4°C in the dark incubator. Human anti-Foxp3-PE was added to the sample and placed in the dark for 30 minutes at room temperature. Cells were washed and assayed by flow cytometry (Calibur, BD, USA) and 10,000 cells in the gate were detected and analysed using Cell Quest software. Cell types were defined as Th1 ($CD4^+IFN\text{-}\gamma^+$), Th2 ($CD4^+IL\text{-}4^+$), Th17 ($CD4^+IL\text{-}17^+$), Treg ($CD4^+CD25^+Foxp3^+$). Absolute numbers of $CD4^+$T subsets were calculated by multiplying percentages of the $CD4^+$T subsets by absolute numbers of $CD4^+$T cells. The phenotypic characteristics of $CD4^+$T cell subsets revealed by dot plot analysis (Supplementary Fig. S1).

Procedures

First all, we compared the $CD4^+$T cell levels in all RP patients and healthy controls without considering the effect of medicines on test results. But 6 of all RP patients have been treated with glucocorticoids or immunosuppressants. Thence, we divided all RP patients into two groups: the untreated RP group (13 patients) and the treated RP group (6 patients). Then we compared the $CD4^+$T cell levels of untreated RP patients, treated RP patients and healthy people in pairs. Analysed levels of $CD4^+$T cells included the absolute numbers and percentages of Th1, Th2, Th17, and Treg cells, as well as the ratios of Th1/Treg, Th2/Treg, Th17/Treg, and Th1/Th2 cells.

In addition, we analysed the discrepancy of absolute number and percentage of Treg cells between RP and RA patients to discover differences of Treg cells in various autoimmune diseases.

ESR and CRP generally reflect the activity of autoimmune diseases. In order to analyse whether the degree of disease activity was related to levels of $CD4^+$T cell subsets, we analysed the correlation between ESR and CRP and the absolute numbers and percentages of $CD4^+$T cell subsets.

Statistical analysis

Continuous variables were expressed as mean ± standard deviation (SD) and median [25^{th}–75^{th} percentile range]; categorical variables were reported as number of occurrences and percentages. The measurement data were compared among controls or RA patients and all RP subjects using the Mann-Whitney U-test (non-normality), and the independent-samples Kruskal-Wallis test (non-normality) was used to compare texture parameters between controls, untreated and treated RP subjects. All analyses were carried out with SPSS v. 22.0 (SPSS, Chicago, IL). In this study, *p*-values (2-tailed) <0.05 denoting statistical significance.

Results

Basic information

Our study recruited 19 patients (8 females and 11 males) who met the inclusion criteria. The average age of patients was 48 years, and the average age at onset was 46 years. The clinical manifestations of these patients were mainly manifested by auricular chondritis (84.21%), and little severe cases showed auricular deformities (10.53%) or hearing loss (21.05%). Others showed arthralgia (63.16%) and ocular lesions, including scleritis (36.84%), conjunctivitis (15.79%), uveitis (15.79%), respiratory symptoms (26.32%) and so on. 6 patients who had received the treatment of immunosuppressants and/or glucocorticoids, 5 had used prednisone at a dose of 10–30 mg/day than three months and 2 had been given disease-modifying antirheumatic drugs (DMARDs) more than six months. Demographic, clinical characteristics and treatment of the study population were presented in Table I.

Laboratory data showed that 78.95% of patients had elevated ESR levels (≥8mg/L) and 47.37% had elevated CRP (levels values >15 mm/h for men and >20 mm/h for women) in 19 pa-

Table I. Demographic and clinical characteristics of 19 patients with RP.

Items		
Sex, female/male	8/11	
Age, mean (S.D.), years	48.00	(10.77)
Age at onset of symptoms, mean (S.D.), years	46.00	(11.39)
Clinical characteristics		
Auricular lesions		
Auricular chondritis	16/19	(84.21%)
Auricle deformities	2/19	(10.53%)
Impaired inner ear		
Tinnitus	3/19	(15.79%)
Hearing loss	4/19	(21.05%)
Joint damage		
Arthralgia	12/19	(63.16%)
Costal chondritis	1/19	(5.26%)
Ocular lesions		
Scleritis	7/19	(36.84%)
Conjunctivitis	3/19	(15.79%)
Uveitis	3/19	(15.79%)
Respiratory symptoms	3/19	(15.79%)
Other symptoms		
Fever	4/19	(21.05%)
Skin rash	2/19	(10.53%)
Medication		
Steroids (prednisone 10-30mg/d)	5/6	(83.33%)
NSAIDs	2/6	(33.33%)
DMARDs (HCQ, LEF, MTX, CTX)	2/6	(33.33%)

NSAIDs: non-steroidal anti-inflammatory drugs; DMARDs: disease-modifying anti-rheumatic drugs; HCQ: hydroxychloroquine; LEF: leflunomide; MTX: methotrexate; CTX: cyclophosphamide.

Table II. Absolute numbers and percentages of CD4+T cells in the study participants.

Variable	RP patients (n=19)	Healthy (n=19)	
PB lymphocyte (cells/μl)	Median (quartile range)	Median (quartile range)	*p*-value
Th1	92.18 (60.09-177.29)	132.39 (77.17-171.19)	0.885
Th2	7.44 (5.13-8.90)	10.19 (7.56-14.18)	0.030*
Th17	7.73 (3.62-14.00)	5.59 (4.23-9.23)	0.470
Treg	22.48 (14.46-33.68)	45.10 (39.63-53.54)	$p<0.001$
Th1/Th2, ratio	13.51 (8.11-22.60)	10.33 (6.15-19.97)	0.297
Th1/Treg, ratio	3.68 (2.26-8.11)	2.06 (1.71-4.06)	0.020*
Th2/Treg, ratio	0.29 (0.22-0.35)	0.21 (0.17-0.29)	0.037*
Th17/Treg, ratio	0.25 (0.19-0.75)	0.14 (0.08-0.19)	$p<0.001$
PB lymphocyte %			
Th1%	14.00 (8.74-20.40)	11.81 (8.75-24.01)	0.563
Th2%	1.00 (0.70-1.42)	1.02 (0.90-1.55)	0.470
Th17%	0.92 (0.70-2.00)	0.68 (0.46-0.92)	0.061
Treg%	3.78 (2.07-4.57)	5.19 (4.69-5.72)	$p<0.001$

tients. IgA levels (>4.53 g/L) raised in 2 patients and IgM (>15.6 g/L) or IgG (>3.04 g/L) was elevated in 1 patient respectively. Only 4 patients were ANA positive and 1 patient was positive for RF, anti-ENA or c-ANCA. Laboratory characteristics of the patients are shown in Supplementary Table S1.

All RP patients display a significant reduction of peripheral Treg cells and the imbalance of Th/Treg cells

To evaluate the status of $CD4^+$T cell subsets in patients with RP, we compared the levels of peripheral $CD4^+$T cell subsets in all RP patients treated or not and healthy controls. The levels of peripheral $CD4^+$T cell subsets in participants were shown on Table II. Compared with healthy controls, all RP patients had lower percentage and absolute number of Treg cells ($p<0.001$, Fig. 1D, H), but not the Th1 and Th17 cells ($p>0.05$, Fig. 1A, C, E, G). The absolute number of Th2 cells in all RP patients was decreased ($p<0.05$, Fig. 1B), but there was no statistical significance about the change of percentage of Th2 cells ($p>0.05$, Fig. 1F). We also analysed the ratios of pro-inflammatory cells to Treg cells in PB of participators. The elevated ratios of Th1/Treg, Th2/Treg and Th17/Treg cells were imbalanced in all RP patients ($p<0.05$, Fig. 2A-C). We are surprised that there was no significant difference in the ratio of Th1/Th2 between RP patients and healthy controls ($p>0.05$, Fig. 2D). So, the main manifestations of $CD4^+$T cell subsets were the decrease in Treg cells and immune imbalance.

Decline of Treg cells and imbalance of Th17/Treg cells in untreated and treated RP patients

In order to rule out the effect of the previous or ongoing treatment on the immune cell levels, we divided all patients into two groups based on whether glucocorticoids or immunosuppressants had been applied. The results were shown in Table III. Absolute numbers and percentages of peripheral Treg cells of untreated and treated RP patients were significantly lower than those in healthy controls ($p<0.001$, Fig. 3D, H). Notably, neither absolute number nor proportion of peripheral Th1, Th2, or Th17 cells was increased in untreated and treated RP patients ($p>0.05$, Fig. 3A-C, E-G). Interestingly, treated RP patients had significantly fewer absolute numbers of peripheral Treg cells than untreated RP patients ($p<0.01$, Fig. 3D). The comparison of the ratio of multiple Th subsets to Treg cells between the three groups was also shown. Due to fewer Treg cells, the ratio of Th1/Tregs in treated RP patients was increased as compared with untreated RP patients ($p<0.05$, Suppl. Fig. S2A), and the ratio of Th17/Tregs in the untreated RP groups and the ratios of Th2/Tregs and Th17/Tregs in

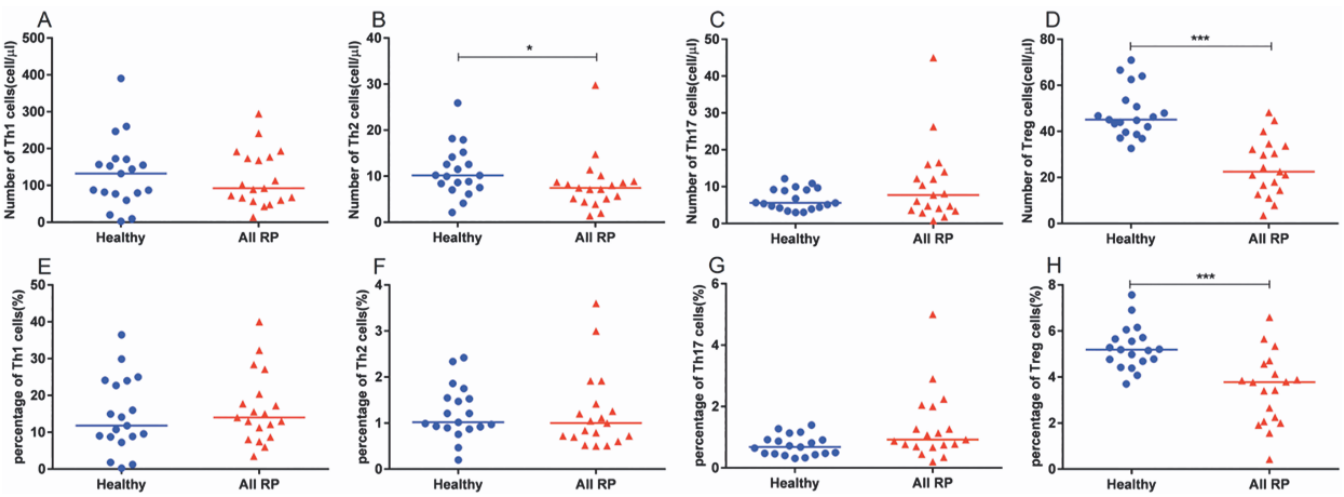

Fig. 1. Characteristics of absolute numbers and percentages of peripheral Th1, Th2, Th17 and Treg cells assessed by flow cytometry in 19 healthy controls and 19 RP patients.

A, **C**, **E**, **G**: There was no statistically significant change in absolute numbers or percentages of Th1 and Th17 cells.

B, **F**: Compared with healthy controls, the absolute number of Th2 cells decreased, but its percentage did not change.

D, **H**: The absolute number and percentage of Treg cells in all RP patients were significantly decreased compared with healthy controls.

All data are presented as medians. Statistical analyses were performed using the Mann-Whitney U-test (non-normality). *$p<0.05$; ***$p<0.001$.

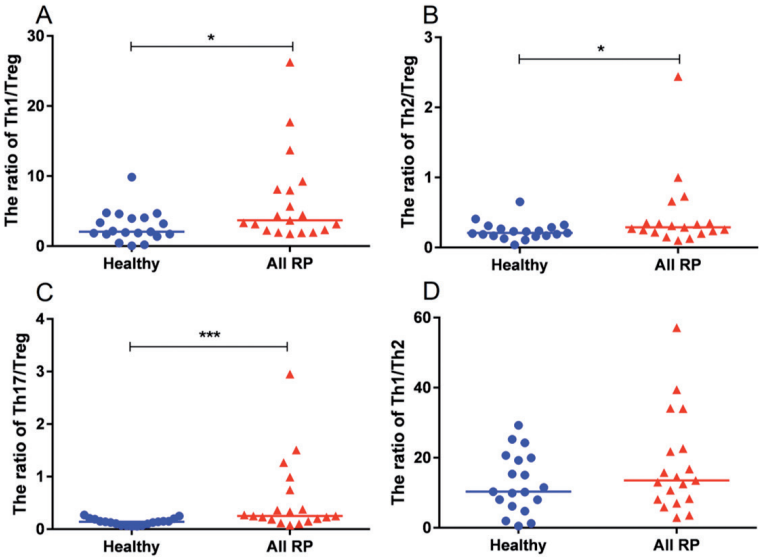

Fig. 2. Imbalances of between Th1, Th2, Th17 and Treg cells in 19 healthy controls and 19 RP patients. **A**-**C**: The ratios of Th1/Treg, Th2/Treg and Th17/Treg cells in all RP patients increased compared with healthy controls. **D**: The ratio of Th1/Th2 cells was not altered significantly.
All data are presented as medians. Statistical analyses were performed using the Mann-Whitney U-test (non-normality). *p<0.05; ***p<0.001.

the treated RP group were significantly higher than in healthy group (p<0.01, Suppl. Fig. S2C; p<0.05, Suppl. Fig. S2B-C). However, the ratio of Th1/Th2 was not significantly changed (p>0.05, Suppl. Fig. S2D). Therefore, the main manifestations of CD4⁺T cell subsets in untreated RP patients were reduced Treg cells and imbalances of Th/Treg cells, especially the imbalance of Th17/Treg, and treated RP patients had lower levels of Treg cells than untreated RP patients.

Difference of Treg cells in peripheral blood between RP and RA patients

Although the absolute number and percentage of peripheral Treg cells in RA and RP patients were lower than those in the healthy control group (p<0.001, Suppl. Fig. S3A-B), there was no statistically significant difference on those between RA and RP patients (p>0.05, Suppl. Fig. S3A-B).

Correlation analysis

ESR and CRP, the common indicators of disease activity, had no significant correlation with the absolute numbers and percentages of Th1, Th2, Th17, and Treg cells (date not shown).

Discussion

In order to better understand the states of CD4⁺T subsets, Th1, Th2, Th17 and Treg cells in blood were analysed in RP patients and healthy controls. The results showed that Treg cells were the only cell subset with a significant difference (a lower absolute number and percentage) in CD4⁺T cell subsets in all patients, including untreated and treated RP patients, as compared with the healthy group. Above results indicate that absolute reduction of circulating Treg cells in untreated RP patients directly associated with pathogenesis of RP. Interestingly, the levels of peripheral Treg cells in treated RP patients was even lower than that in the untreated cases. Conventional immunosuppressants and glucocorticoids (6) could affect the level of peripheral Treg cell, but more sample sizes are needed to confirm this result.

The main role of Treg cells is to suppress excessive immune activation and maintain immune homeostasis. Autoimmune diseases were caused by a deficiency in immune tolerance due to the decrease of Treg cells (7). Studies found that the dysfunction and reduction of Treg were linked to the pathogenesis of RA and SLE (8, 9). Peripheral Treg cells in RP patients was also decreased as in RA patients in our study, but there was no significant difference about Treg cells in both. The result confirmed that peripheral Treg cells were reduced in multiple autoimmune diseases again, but differences of Treg cells between different disease species need to be explored further. Therefore, we speculated that Treg cells decrease may be also play a major role in the pathogenesis of RP.

Table III. Absolute numbers and percentages of CD4⁺T cells in healthy controls, untreated RP and treated RP patients.

Variable	Untreated RP (n=13)	Treated RP (n=6)	Healthy (n=13)	
PB lymphocyte (cells/μl)	Median (quartile range)	Median (quartile range)	Median (quartile range)	p-value
Th1	113.18 (67.27-192.70)	74.56 (35.72-119.85)	87.74 (69.78-164.05)	0.369
Th2	7.44 (5.43-9.53)	6.20 (3.44-10.13)	9.98 (7.29-12.57)	0.191
Th17	8.12 (4.20-16.27)	4.44 (2.78-11.29)	5.68 (4.61-9.45)	0.315
Treg	30.45 (21.13-37.31)	11.82 (6.82-19.14)	46.33 (40.36-58.04)	p<0.001*
Th1/Th2, retio	15.78 (8.22-28.30)	12.12 (5.98-20.72)	11.51 (5.45-20.33)	0.504
Th1/Treg, retio	3.68 (2.30-6.84)	6.22 (1.87-16.85)	1.95 (1.55-4.01)	0.051
Th2/Treg, retio	0.27 (0.18-0.34)	0.50 (0.24-1.16)	0.20 (0.15-0.27)	0.023*
Th17/Treg, retio	0.25 (0.20-0.56)	0.31 (0.14-1.69)	0.14 (0.11-0.18)	0.009*
PB lymphocyte %				
Th1%	15.57 (10.51-23.76)	11.72 (7.43-18.35)	10.76 (8.01-23.45)	0.416
Th2%	1.00 (0.65-1.67)	0.95 (0.67-1.58)	1.02 (0.83-1.50)	0.918
Th17%	1.07 (0.71-2.15)	0.83 (0.58-1.45)	0.72 (0.49-1.03)	0.269
Treg%	3.80 (3.03-5.03)	2.04 (1.29-4.03)	5.28 (4.74-5.89)	0.001*

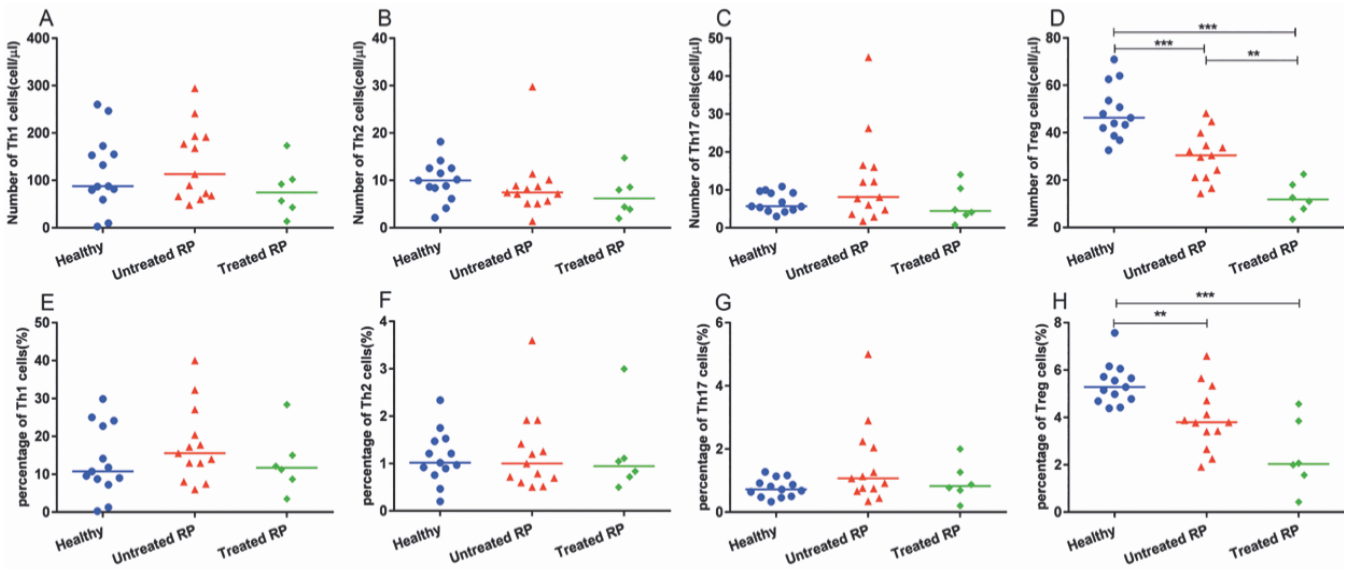

Fig. 3. Relationship between CD4+T cell subsets and treatment. All patients were divided into untreated RP group (n=13) and treated RP group (n=6) based on whether they had been treated with glucocorticoids and immunosuppressants.
A-**C**, **E**-**G**: There was no statistically significant change in absolute numbers or percentages of Th1, Th2, and Th17 cells.
D, **H**: The absolute number and the percentage of Treg cells in untreated and treated RP patients significantly decreased compared with healthy controls, and treated RP patients had lower absolute number of Treg cells than untreated RP patients.
All data are presented as medians. Statistical analyses were performed using the independent-samples Kruskal-Wallis test (non-normality).
$^{**}p<0.01$; $^{***}p<0.001$.

Treg cells account for 5% of circulating CD4$^+$T cells, which can be identified by lineage labelling fork box protein P3 (FOXP3) (7). The defect of development or function of Treg cells leaded to an uncontrolled immune response and tissue destruction to induce the occurrence of autoimmune diseases (7). There may be many reasons for the decrease in Treg cells in PB of patients with RP. Absolute numbers of circulating Treg cells in RP patients may be reduced due to their excessive consumption under extreme inflammatory conditions. The survival of circulating Treg cells required exogenous IL-2, which they could not produce them by themselves (10). Since lose dose IL-2 has been used to expand Treg cells in many autoimmune diseases (4, 11, 12), its role remains to study.

In addition, the absolute number of Th2 cells in all RP patients was lower than that in healthy people while no significant difference between untreated and treated RP patients was found. Simultaneously, neither the absolute number nor proportion of Th1 and Th17 cells were increased in all RP patients. Therefore, the reduction of Treg cells was the main change of peripheral CD4$^+$T cell subsets in patients with RP. Shimizu (13) also found that the function of activated regulatory T cells is impaired, and IL-10 secreted by Treg cells is reduced in RP. Our finding suggested that an absolute reduction in the number of circulating Treg cells may contribute to the functional impairment.

Previous studies suggested that the pathogenesis of RP is mainly caused by Th1/Th2 imbalance (1), which was different from our findings. In our study, it was found that the ratios of Th1/Treg, Th2/Treg and Th17/Treg cells were elevated in RP patients. It is worth noting that abnormal ratios of Th/Tregs might be caused by the reduced absolute number of Treg cells. Th17 cells and Treg cells have opposite effects in the inflammatory response, and the imbalance in the numbers of them have been found to be involved in the pathogenesis of various autoimmune diseases (14-17). Interestingly, the imbalance between Th17 and Treg cells also appeared in peripheral blood of new-onset RP patients. Most of previous studies reported only the relative changes (proportion) of peripheral lymphocytes in autoimmune diseases. However, we found significant changes only in absolute numbers of lymphocyte subpopulations rather than percentages of them. Therefore, both relative and absolute levels should be assessed to evaluate the changes of these cells. In this study, we showed for the first time that the status of absolute numbers and imbalance of peripheral CD4$^+$T cell subsets in RP patients, associating the most possible pathogenesis. However, the number of patients in our study is limited and few RP patients were diagnosed every year. Further efforts in larger study populations are demanded to address in a more accurate fashion the value and dynamics of significant contributors of immune response in RP. The function of Th cells and Treg cells in RP also need to be studied further to understand the immunopathological mechanism accurately.

In summary, the reduction in the absolute number of Treg cells and imbalance with Th cells, especially the imbalance of Th17/Treg cells, play an important role in the pathogenesis of RP. These findings revealed the possible underlying pathogenesis of RP patients and provided a new direction for RP treatment by increasing the absolute number of Treg cells to improved immune tolerance. Additional studies are needed to further explore the specific mechanism of reduction of Treg cells in RP patients.

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