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Expression of T follicular helper lymphocytes with different subsets and analysis of serum IL-6, IL-17, TGF-β and MMP-3 contents in patients with rheumatoid arthritis

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Abstract. – OBJECTIVE: To investigate the expression levels of T follicular helper (Tfh) with different subsets in patients with rheumatoid arthritis (RA) and their serum interleukin-6 (IL-6), interleukin-17 (IL-17), transforming growth factor-β (TGF-β) and matrix metalloproteinase 3 (MMP-3) contents.

PATIENTS AND METHODS: The medical records of 45 RA patients in the Department of Rheumatology and Immunology in the First Affiliated Hospital of Chengdu Medical College from January 2016 to April 2018 were retrospectively analyzed. They were divided into the RA high activity group (24 cases, group A) (DAS28 score ≥ 5.0) and RA low activity group (21 cases, group B) (3.2 < DAS28 score < 5.0). At the same time, 20 healthy subjects were selected as a control group. Flow cytometry was used to detect the expression levels of Tfh1, Tfh2 and Tfh17, enzyme-linked immunosorbent assay to detect serum IL-6, IL-17, TGF-β and MMP-3 concentrations. The correlation of Tfh cells with IL-6, IL-17, TGF-β and MMP-3 was analyzed.

RESULTS: Those of peripheral blood mononuclear cell (PBMC) Tfh2 and Tfh17 cells were significantly higher in group A than those in group B ($p < 0.05$). Compared with the control group, the concentrations of serum IL-6, IL-17 and MMP-3 significantly increased ($p < 0.001$), but that of serum TGF-β markedly decreased in group A and group B ($p < 0.01$). The concentrations of serum IL-6, IL-17 and MMP-3 were remarkably higher in group A than those in group B ($p < 0.001$), but that of serum TGF-β was significantly lower in group A than that in group B ($p < 0.001$). The expression level of PBMC Tfh2 cells, PBMC Tfh17 cells was positively correlated with serum IL-6, IL-17 and MMP-3. The expression levels of Tfh2 and Tfh17 cells are positively correlated with serum IL-6, IL-17 and MMP-3 concentrations, negatively correlated with serum TGF-β concentration.

CONCLUSIONS: Tfh2 and Tfh17 are expected to be new targets for immunotherapy in RA patients.

Key Words:
Rheumatoid arthritis, Tfh2, Tfh17, IL-6, IL-17, TGF-β, MMP-3.

Introduction

Rheumatoid arthritis (RA), a common systemic, heterogeneous and autoimmune disease in humans, is mainly characterized by chronic, invasive and symmetrical small arthritis, which can affect multiple organs such as lung, kidney and heart[1]. At present, the pathogenesis of RA has not been elucidated. Most scholars believe that it is due to immune systemic disorders such as the abnormal activation of T lymphocyte subsets and the imbalance of B lymphocytes caused by genetically susceptible individuals under the influence of various exogenous infections[2,3]. As a new type of $CD4^+$T cell subset newly discovered in recent years, the main function of T follicular helper (Tfh) is to assist B lymphocytes to maintain and induce the body's immune response[4]. According to the difference in (chemokine receptor 3) CXCR3 and chemokine receptor 6 (CCR6) chemokine receptors on the surface of Tfh cells, Tfh cells can be divided into three subsets, namely Tfh1 ($CCR6^-CXCR3^+$Tfh), Tfh2

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(CCR6$^-$CXCR3$^-$Tfh) and Tfh17 (CCR6$^+$CXCR3$^-$Tfh)[5]. Previous studies have shown that the expression of Tfh cell subsets is closely related to diseases such as autoimmune thyroid disease and systemic lupus erythematosus[6,7]. Tfh cells secrete immune inflammatory factors such as in-terleukin-6 (IL-6) and interleukin-17 (IL-17). The two one interacts with transforming growth factor-β (TGF-β) to pro-duce a large number of plasmablasts, involved in the body's immune response[2,8]. The former two, that are key factors in the body's immune regulation, can promote cell differentiation, stimulate cell growth and regulate the immune response, involved in the body's stress response[9]. Transforming growth factor-β (TGF-β) is an important osteoblast-promoting factor that inhibits osteoclast formation and promotes osteoblast development, promoting bone formation[10]. Matrix metalloproteinase 3 (MMP-3), an important member of the MMPs family, not only degrades bone and cartilage, damaging the joints of RA patients, but also induces other MMPs to exert biological effects to destroy human joints[11]. In this study, flow cytometry was used to detect the expression levels of Tfh1, Tfh2 and Tfh17 in Tfh cells subsets of RA patients, enzyme-linked immunosorbent assay (ELISA) to detect serum IL-6, IL-17, TGF-β and MMP-3 concentrations. The correlation of Tfh1, Tfh2 and Tfh17 with the occurrence and development of RA and its mechanism of action were observed, to provide new targets for the treatment of RA patients.

Patients and Methods

Patients

The medical records of 45 RA patients in the Department of Rheumatology and Immunology in The First Affiliated Hospital of Chengdu Medical College from January 2016 to April 2018 were retrospectively analyzed. The degree of disease activity of RA patients was differentiated according to RA disease activity (DAS28) score[12], with 24 patients in RA high activity group (group A) (DAS28 score ≥5.0) and 21 patients in RA low activity group (group B) (3.2 < DAS28 score <5.0). Group A included 5 males and 19 females, with an average age of (49.85ed t22) years. Group B included 7 males and 14 females, with an average age of (48.27 ± 13.81) years. At the same time, 20 healthy subjects were selected as a control group, including 4 males and 16 females, with an average age of (48.21 ± 12.73) years.

Inclusion and Exclusion Criteria

Inclusion criteria: patients were included in line with the RA classification and diagnosis criteria developed by the European League against Rheumatism (EULAR) combined with the American College of Rheumatology (ACR) in 2015[13]. This study was approved by the Ethics Committee of The First Affiliated Hospital of Chengdu Medical College. Subjects and their families signed the informed consent. Exclusion criteria: patients with severe liver, kidney and hematopoietic dysfunction were excluded; lactating or pregnant women; patients with other rheumatic diseases such as osteoarthritis, spondyloarthritis, systemic lupus erythematosus; patients with mental illness or family history of mental illness.

Detection of Tfh Cell Subclass

4 mL of peripheral venous blood was extracted from subjects in a fasting state, placed in a heparin sodium anticoagulant tube and centrifuged to obtain the supernatant. The supernatant was diluted with Phosphate-Buffered Saline (PBS) buffer and placed in a centrifuge tube with an equal amount of lymphocyte separation solution at the bottom. After centrifugation at 2000 rpm for 20 min, the middle white membrane layer was taken to isolate peripheral blood mononuclear cell (PBMC). The appropriate amount of PBS buffer was added, centrifuged at 2000 rpm for 20 min and frozen in liquid nitrogen with supernatant discarded. During the experiment, PBMC was taken and transferred to a flow tube, with cell concentration adjusted to 5×10^5 cells/mL. In the environment of 4°C, the antibody AF700-CD3 (Shanghai Biotechnology Co., Ltd., Shanghai, China), PECF-594-CD4 (Shanghai Biotechnology Co., Ltd., Shanghai, China), AF-647-CXCR5 (Shanghai Biotechnology Co., Ltd., Shanghai, China), PECF-594-CXCR3 (BD Biosciences, Franklin Lakes, NJ, USA) and BV-650-CCR6 (Beijing Biotechnology Co., Ltd., Beijing, China) were added. After standing for 30 min, centrifugation was performed at 3000 rpm for 5 min, with supernatant discarded. 5 mL of PBS buffer was added, shaken and mixed, centrifuged at 3000 rpm for 5 min, with supernatant discarded and repeated once. Flow cytometry (Shanghai Biotechnology Co., Ltd., Shanghai, China) was used to detect the fluorescence intensity of the stained cell marker.

Detection of Serum IL-6, IL-17, TGF-β and MMP-3 Concentrations

5 mL of venous blood was extracted from subjects in a fasting state, placed in a vacuum tube without anticoagulant, centrifuged to separate serum and stored in a -20°C cryogenic refrigerator for use. ELISA was used to detect serum IL-6, IL-17, TGF-β and MMP-3 concentrations, with reference to the ELISA kit instructions of human IL-6, IL-17, TGF-β (Wuhan Technology Co., Ltd., Wuhan, China) and human MMP-3 (Shanghai Trading Co., Ltd., Shanghai, China). The sample to be tested and the kit were taken out from the refrigerator 30 min in advance to balance the room temperature, with a blank well, a standard well and a sample well respectively. 100 μL of the sample dilution was added to the blank well, and 100 μL of the standard or the sample to be tested were added to other wells, respectively, mixed gently, covered with a membrane and incubated at 37°C for 120 min; the liquid discarded from each well and dried. 100 μL of biotin-labeled antibody working solution was added to each well, mixed gently and incubated at 37°C for 60 min; the liquid was discarded from each well, dried and repeatedly washed 3 times. 100 μL of peroxidase-labeled avidin working solution was added to each well and incubated at 37°C for 60 min; the liquid was discarded from each well, dried and repeatedly washed 3 times. 90 μL of substrate solution was added to each well in sequence, illuminating at 37°C for 15 min in the dark. 50 μL of stop solution was added to each well in sequence to terminate the reaction. DNM-9602A automatic enzyme-mark analyzer (Beijing Pulang New Technology Co., Ltd., Beijing, China) was used to detect the optical density (OD) value of each well at a wavelength of 450 nm. The concentrations of IL-6, IL-17, TGF-β and MMP-3 were calculated.

Statistical Analysis

SPSS 20.0 (Beijing Sichuangweida Information Technology Co., Ltd., Beijing, China) was used for statistical analysis. The measurement data were expressed as mean ± standard deviation ($\bar{x} \pm sd$). One-way analysis of variance was used for the comparison of mean among multiple groups and the post-hoc test was the LSD test. The count data were expressed as a percentage. The chi-square test was used for the comparison of the count data among groups and Spearman test for the correlation analysis. When $p < 0.05$, the difference is statistically significant.

Results

Baseline Data of the Three Groups

The difference was not statistically significant among group A, B and control group in the clinical baseline data such as sex, age, blood glucose (Glu), blood urea (BUN), serum creatinine (Scr), β2 microglobulin (β2-MG), blood uric acid (UA), alanine aminotransferase (ALT)), r-glutamyltransferase (r-GT) and aspartate aminotransferase (AST) (all $p > 0.05$). See Table I.

Expressions of Three Groups of PBMC Tfh Cell Subsets

The expression levels of PBMC Tfh1 cells in group A, B and control group were (30.57 ± 4.59) %, (33.47 ± 4.58) % and (32.19 ± 5.31) %, respectively. Those of Tfh2 cells were (45.58 ± 9.57) %, (39.87 ± 6.47) % and (35.18 ± 4.38) %, respectively.

Table I. Baseline data of three groups [n (%)]/($\bar{x} \pm sd$).

Category	Group A (n = 24)	Group B (n = 21)	Control group (n = 20)	F/χ^2	p
Sex				1.275	0.539
Male	5 (20.83)	7 (33.33)	4 (20.00)		
Female	19 (79.17)	14 (66.67)	16 (80.00)		
Age (years)	49.85 ± 14.22	48.27 ± 13.81	48.21 ± 12.73	0.105	0.900
Glucose (mmol/L)	6.01 ± 0.94	5.78 ± 0.87	6.03 ± 1.27	0.383	0.683
BUN (mmol/L)	4.13 ± 1.13	4.22 ± 1.02	4.19 ± 0.93	0.321	0.726
Scr (μmol/L)	82.14 ± 11.75	86.47 ± 14.36	82.96 ± 13.57	0.660	0.520
β2-MG (mg/L)	3.41 ± 0.37	3.38 ± 0.42	3.43 ± 0.35	0.089	0.914
UA (μmol/L)	153.63 ± 20.41	154.85 ± 22.95	149.63 ± 19.74	0.344	0.710
ALT (U/L)	62.47 ± 13.24	63.58 ± 15.27	61.48 ± 14.52	0.110	0.895
r-GT (U/L)	44.52 ± 11.52	41.96 ± 13.79	40.58 ± 10.23	0.623	0.539
AST (U/L)	19.63 ± 6.47	18.57 ± 7.15	19.01 ± 6.85	0.138	0.871

Those of Tfh17 cells were (31.57 ± 7.64) %, (24.63 ± 6.41) % and (18.69 ± 5.36) %, respectively. The expression levels of PBMC Tfh2 and Tfh17 cells were significantly higher in group A and B than those in the control group (t = 4.478, $p < 0.001$; t = 2.704, $p = 0.010$; t = 6.344, $p < 0.001$; t = 3.547, $p = 0.001$). Those of PBMC Tfh2 and Tfh17 cells were significantly higher in group A than those in group B (t = 2.310, $p = 0.025$; t = 3.478, $p = 0.001$). The difference was not significant in the expression level of PBMC Tfh1 cells among group A, B and control group ($p > 0.05$). (Figure 1).

Concentrations of Serum IL-6, IL-17, TGF-β and MMP in the Three Groups

The concentrations of serum IL-6 in group A, B and control group were (91.25 ± 16.75) pg/mL, (73.41 ± 10.67) pg/mL and (59.57 ± 4.86) pg/mL, respectively. Those of serum IL-17 were (82.63 ± 17.32) pg/mL, (56.78 ± 8.74) pg/mL and (35.87 ± 4.51) pg/mL, respectively. Those of serum TGF-β were (13.67 ± 5.79) ng/mL, (24.49 ± 6.52) ng/mL and (31.52 ± 9.74) ng/mL, respectively. Those of serum MMP-3 were (83.52 ± 11.63) ng/mL, (57.63 ± 8.57) ng/mL and (46.67 ± 8.26) ng/mL, respectively. Compared with the control group, the concentrations of serum IL-6, IL-17 and MMP-3 significantly increased (t = 8.163, $p < 0.001$; t = 5.299, $p < 0.001$; t = 11.730, $p < 0.001$; t = 9.553, $p < 0.001$; t = 11.880, $p < 0.001$; t = 4.166, $p < 0.001$), but that of serum TGF-β markedly decreased (t = 7.532, $p < 0.001$; t = 2.729, $p = 0.009$) in group A and group B. Those of serum IL-6, IL-17 and MMP-3 were remarkably higher in group A than those in group B (t = 4.190, $p < 0.001$; t = 6.180, $p < 0.001$; t = 8.396, $p < 0.001$), but that of serum TGF-β was significantly lower in group A than that in group B (t = 5.897, $p < 0.001$). See Figure 2.

Correlation of Expression Levels of PBMC Tfh2 and Tfh17 Cells With Serum IL-6, IL-17, TGF-β and MMP-3 Concentrations

The results of the Spearman test showed that the expression level of PBMC Tfh2 cells was positively correlated with serum IL-6, IL-17 and MMP-3 (r = 0.433, $p = 0.003$; r = 0.730, $p < 0.001$; r = 0.505, $p < 0.001$), negatively correlated with serum TGF-β concentration (r = -0.475, $p = 0.001$). See Figure 3. The expression level of PBMC Tfh17 cells was positively correlated with serum IL-6, IL-17 and MMP-3 (r = 0.478, $p < 0.001$; r = 0.708, $p < 0.001$; r = 0.520, $p < 0.001$), negatively correlated with serum TGF-β concentration (r=-0.371, $p = 0.012$). See Figure 4.

Discussion

As an autoimmune disease pathologically characterized by joint destruction and progressive synovial inflammation, RA, one of the common rheumatic diseases in the clinic, has a higher incidence in humans[14]. Occurring in any age group,

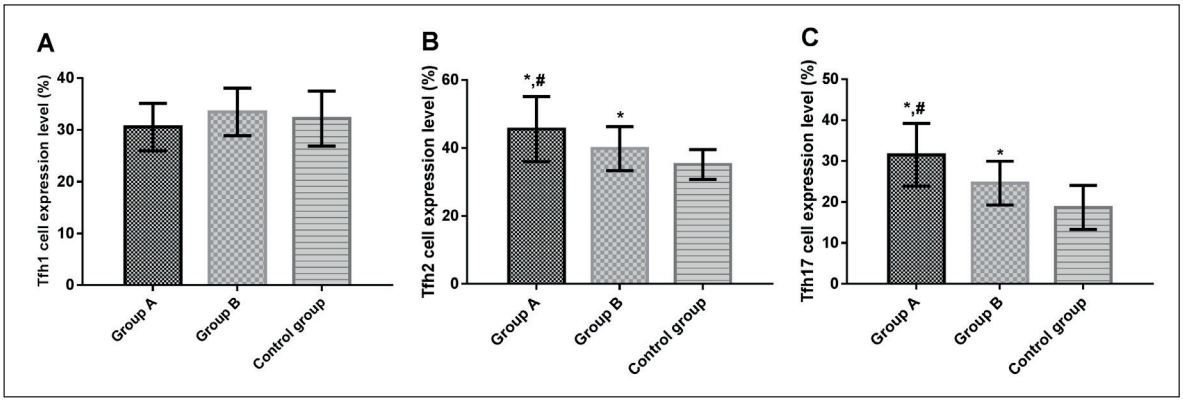

Figure 1. Result comparison of the expression levels of PBMC Tfh1, Tfh2 and Tfh17 cells among group A, group B and control group. The results of flow cytometry showed that the difference was not significant in the expression level of PBMC Tfh1 cells among group A, B and control group ($p > 0.05$). ***A,*** The expression levels of PBMC Tfh2 cells were markedly higher in group A and group B than those in the control group ($t = 4.478$, $p < 0.001$; $t = 2.704$, $p = 0.010$). That of PBMC Tfh2 cells was remarkably higher in group A than that in group B ($t = 2.310$, $p = 0.025$) ***B,*** Those of PBMC Tfh17 cells were significantly higher in group A and group B than those in the control group ($t = 6.344$, $p < 0.001$; $t = 3.547$, $p = 0.001$). That of PBMC Tfh17 cells was markedly higher in group A than that in group B ($t = 3.478$, $p = 0.001$) ***C***. Note: *indicates that compared with the control group, $p < 0.01$. # indicates that compared with group B, $p < 0.05$.

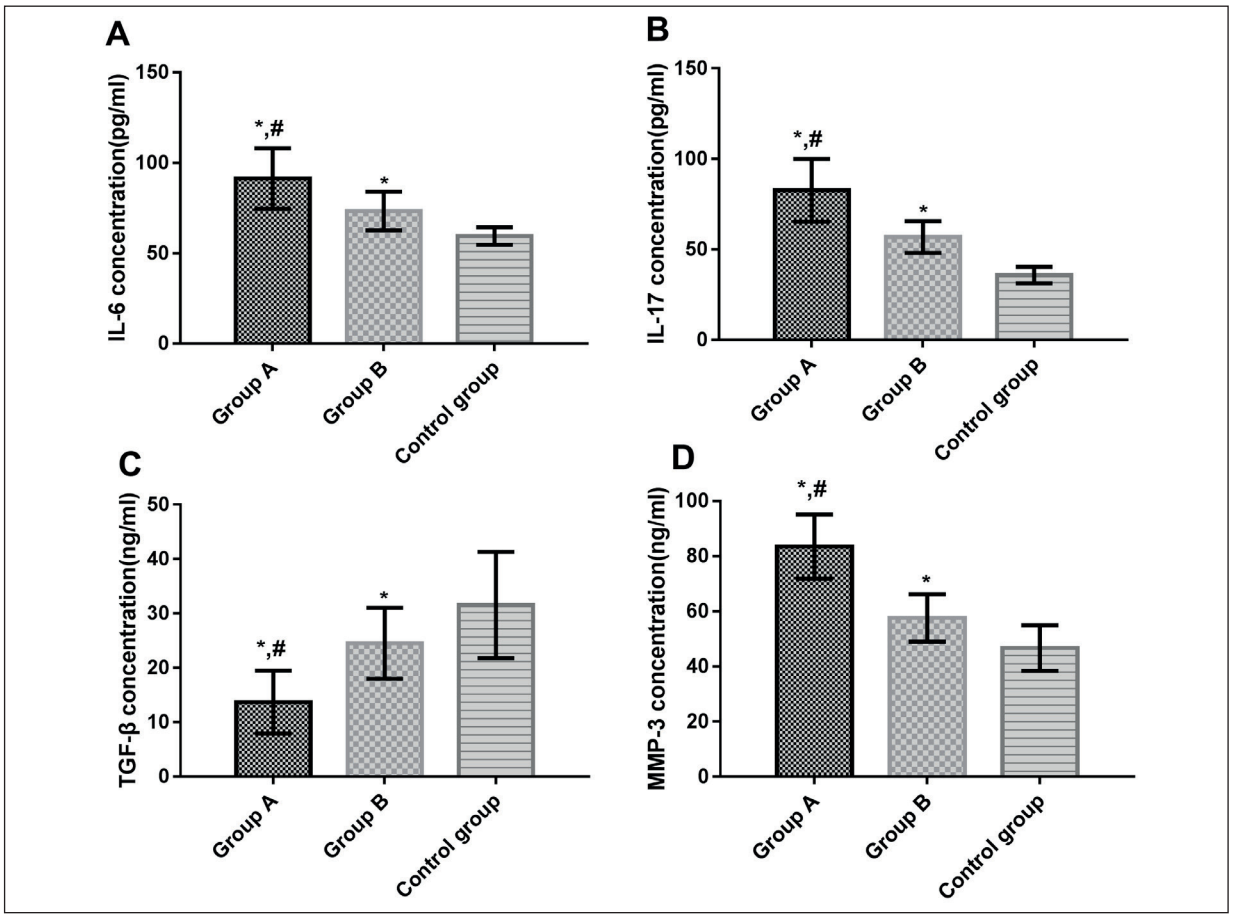

Figure 2. Result comparison of serum IL-6, IL-17 and TGF-β concentrations among group A, group B and control group. The results of ELISA showed that, compared with the control group, the concentrations of serum IL-6 significantly increased in group A and group B ($t = 8.163$, $p < 0.001$; $t = 5.299$, $p < 0.001$), and that of serum IL-6 was significantly higher in group A than that in group B ($t = 4.190$, $p < 0.001$) ***A,*** Compared with the control group, those of serum IL-17 markedly increased in group A and group B ($t = 11.730$, $p < 0.001$; $t = 9.553$, $p < 0.001$), and that of serum IL-17 was significantly higher in group A than that in group B ($t = 6.180$, $p < 0.001$) ***B,*** Compared with the control group, those of serum TGF-β significantly decreased in group A and group B ($t = 7.532$, $p < 0.001$; $t = 2.729$, $p = 0.009$), and that of serum TGF-β was remarkably lower in group A than that in group B ($t = 5.897$, $p < 0.001$) ***C,*** Compared with the control group, those of serum MMP-3 significantly increased in group A and group B ($t = 11.880$, $p < 0.001$; $t = 4.166$, $p < 0.001$), and that of serum MMP-3 was markedly higher in group A than that in group B ($t = 8.396$, $p < 0.001$) ***D.*** Note: * indicates that compared with the control group, $p < 0.01$. # indicates that compared with group B, $p < 0.001$.

its incidence increases with age, which in women is 2.3 times that in men, lack of effective preventive measures and treatment options for it in clinic[15]. Although the etiology and pathogenesis of RA have not yet been elucidated, most scholars believe that its pathogenesis is closely related to the body's immune system disorder[16,17].

As a new type of $CD4^+$T cell subset discovered in recent years, Tfh cells can assist B lymphocytes to induce an antibody response, promoting B lymphocytes with high affinity to differentiate into long-acting memory cells and plasmocytes. They also produce immune globulin and participate in the conversion process of immune globulin species, to maintain the humoral immune response[18,19]. They can be divided into three different Tfh cell subsets, namely Tfh1, Tfh2 and Tfh17 cells. The latter two are capable of inducing naive B cells to produce antibodies and perform type switching, but the former one does not[20]. Changes in the balance of Tfh cell subsets may lead to autoimmune diseases in humans. Tfh hyperfunction causes B cells to be over-activated, to differentiate into memory B cell nucleoplasm cells and secrete high-affinity autoantibodies that induce pathological damage, thus resulting in pathological autoimmune response in the body. The occurrence of various immune diseases such

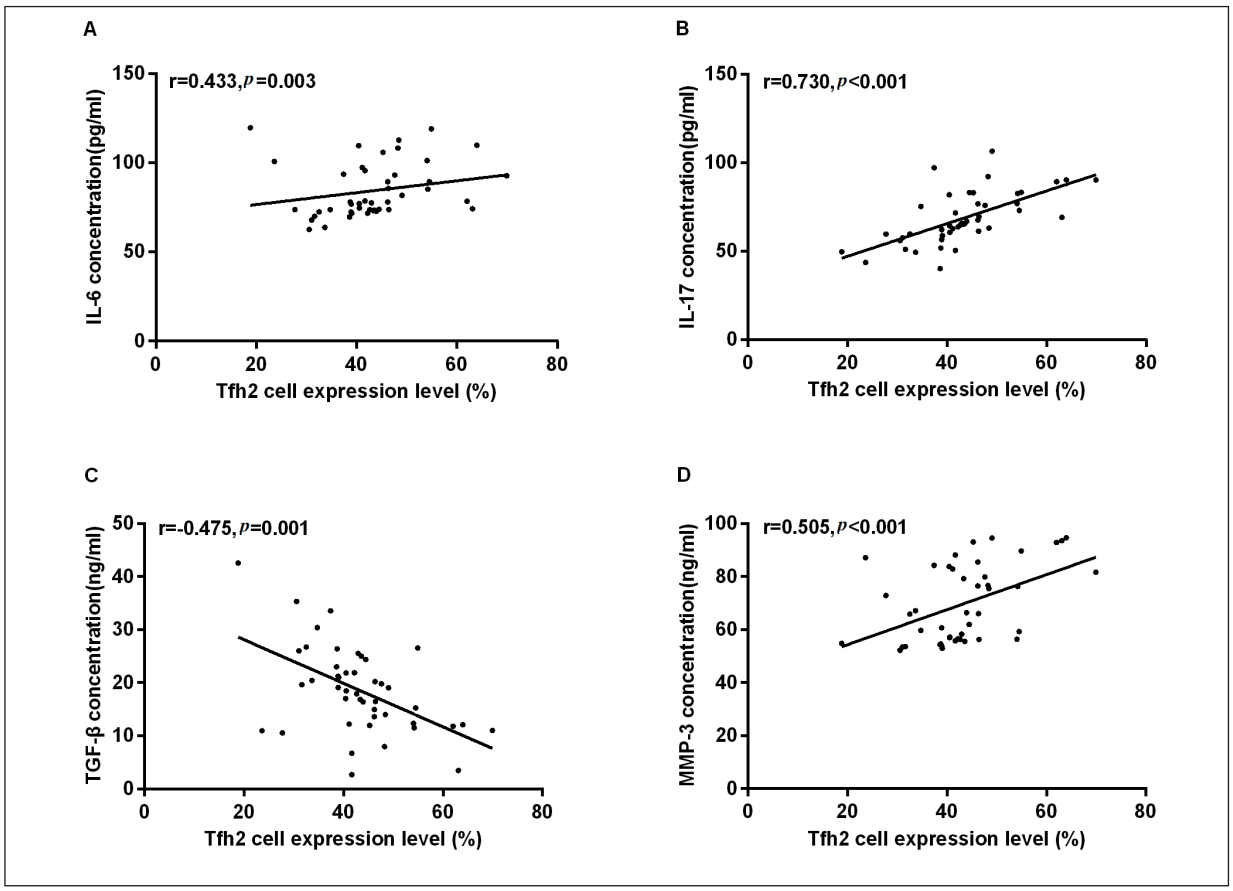

Figure 3. Correlation of expression level of PBMC Tfh2 cells with serum IL-6, IL-17, TGF-β and MMP-3 concentrations. The results of Spearman test showed that the expression level of PBMC Tfh2 cells was positively correlated with serum IL-6, IL-17 and MMP-3 (r = 0.433, p = 0.003; r = 0.730, $p < 0.001$; r = 0.505, $p < 0.001$), negatively correlated with serum TGF-β concentration (r = -0.475, p = 0.001).

as systemic lupus erythematosus and myasthenia gravis are mediated[21]. The peripheral blood Tfh cell subsets of RA patients were studied. The results showed that the expression levels of PBMC Tfh2 and Tfh17 cells were significantly higher in group A and group B than those in the control group, and those of PBMC Tfh2 and Tfh17 cells were significantly higher in group A than those in group B, indicating that Tfh2 and Tfh17 cells are involved in the occurrence and development of RA to a certain extent. As the disease aggravates, their levels in RA patients gradually increase, with a more severe immune disorder in the body. Masaki et al[22] believe that Tfh cell count in young dermatomyositis patients is heavily biased toward Tfh2 and Tfh17 cells, and the disease activity is closely related to the increase in circulating plasmablasts. Che et al[23] suggest that Tfh cell subsets of Tfh2 and Tfh17, as predictors evaluating the progress of Guillain-Barré syndrome, may promote autoantibody-related immune responses, similar to this work.

IL-6 family, an important inflammatory factor in RA, can induce the acute phase protein of liver synthesis, promoting B lymphocytes to differentiate and proliferate and secrete antibodies. Under the action of TGF-β, IL-6 can up-regulate T lymphocytes to differentiate into Th17 cells. Inhibiting TGF-β, it can also reduce the differentiation of regulatory T lymphocytes, with its pro-inflammation ability in cellular immunity higher than anti-inflammation ability[24]. Produced by differentiated Th17 cells, IL-17 can induce activated T lymphocytes, which promotes synovial cells to secrete various inflammatory cytokines such as IL-6 and IL-8. It also up-regulates the expressions of MMP-3 in synovial fibroblasts and chondrocytes[25]. In the early stage of RA patients, a large number of fibroblasts and chondrocytes

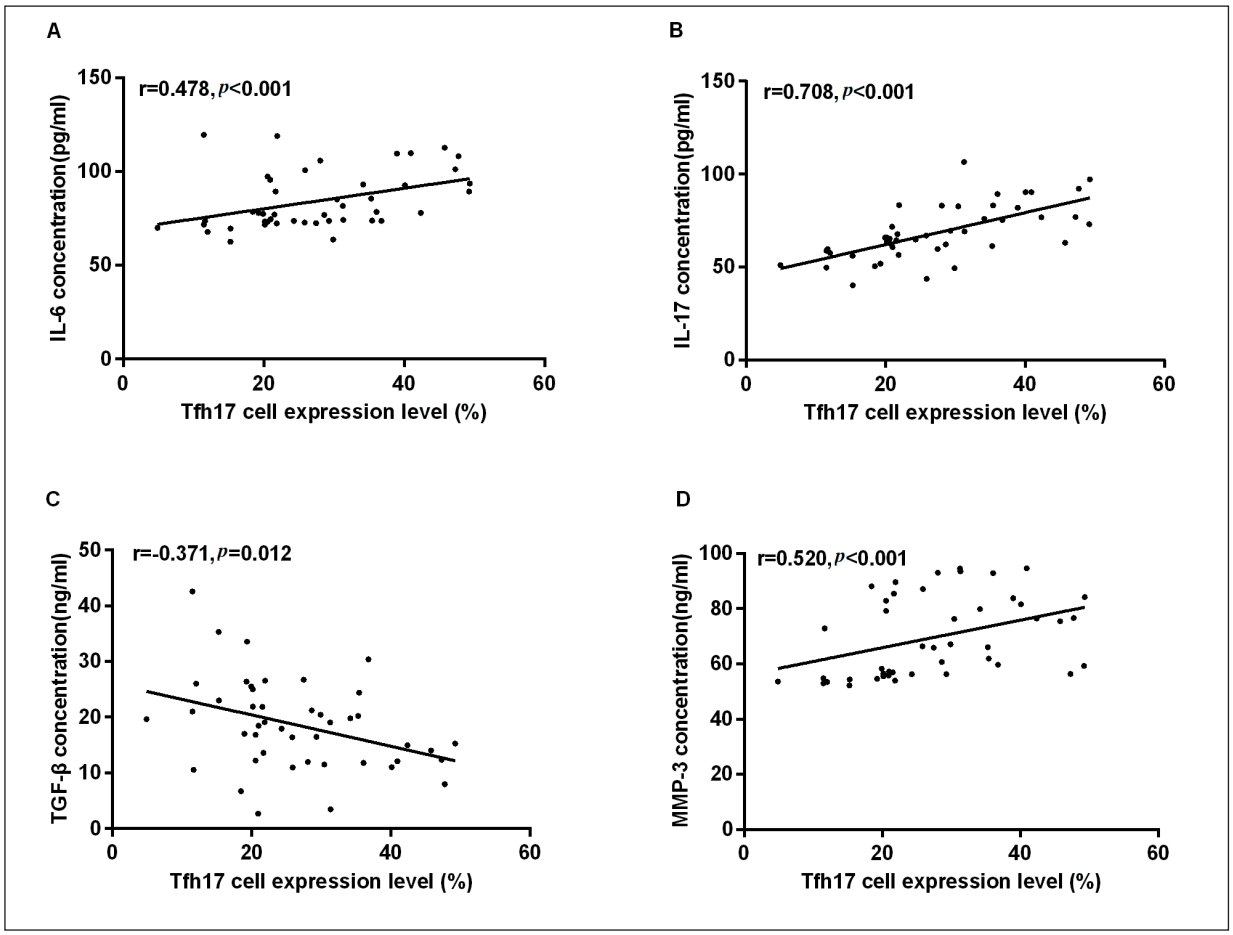

Figure 4. Correlation of expression level of PBMC Tfh17 cells with serum IL-6, IL-17, TGF-β and MMP-3 concentrations. The results of Spearman test showed that the expression level of PBMC Tfh17 cells was positively correlated with serum IL-6, IL-17 and MMP-3 ($r = 0.478$, $p < 0.001$; $r = 0.708$, $p < 0.001$; $r = 0.520$, $p < 0.001$), negatively correlated with serum TGF-β concentration ($r = -0.371$, $p = 0.012$).

secrete MMP-3, significantly up-regulating the MMP-3 in the blood or synovial fluid to form a pannus, thereby causing erosion in articular cartilage tissue. As a result, pathological changes occur in bone tissue[26]. The up-regulation of TGF-β expression can stimulate the increase of articular chondrocytes, reducing the damage to cartilage tissue in the body, to delay the course of the disease[27]. The results of this study showed that, compared with the control group, the concentrations of serum IL-6, IL-17 and MMP-3 significantly increased, but that of serum TGF-β markedly decreased in group A and B. The concentrations of serum IL-6, IL-17 and MMP-3 were significantly higher in group A than those in group B, but that of serum TGF-β was remarkably lower in group A than that in group B. It is suggested that involved in the inflammatory response and joint destruction process of RA, IL-6, IL-17, TGF-β and MMP-3 can reflect RA disease activity. Further studies have shown that the expression levels of PBMC Tfh2 and Tfh17 cells were positively correlated with serum IL-6, IL-17 and MMP-3 concentrations, negatively correlated with serum TGF-β concentration. It is suggested that Tfh2 and Tfh17 cells in Tfh cell subsets can reflect the disease activity status of RA patients. As the disease progresses, the aggravated disorder degree of them may affect the biological effects of RA, further promoting the development of it. A large number of inflammatory cytokines are released during the progression, and inflammatory factors infiltrate the joint tissue, which hinders the development of osteoblast and promotes pathological chang-

es in bone tissue, exacerbating joint destruction. The study of Chen et al[28] has shown that Tfh effect memory cells, related to the degree of RA disease activity, are valuable markers of active RA. The increased Tfh2 and related inflammatory cytokines may be involved in the occurrence and development of RA. Therefore, Tfh2 and Tfh17 may become new targets for immunotherapy in RA patients.

In the work, subjects were screened in strict accordance with the inclusion and exclusion criteria. There was no difference among group A, B and control group in sex, age, Glucose, BUN, Scr, β2-MG, UA, ALT, r-GT and AST, which ensured the rigor and reliability of this study. The sample size of this research was small, and changes in the expression levels of Tfh2 and Tfh17 cells in peripheral blood of RA patients after medication were not observed, so there were certain limitations. In future works, the sample size should be expanded, and the study time should be extended. The expression levels of Tfh2 and Tfh17 cells in peripheral blood of RA patients before and after treatment should also be observed.

Conclusions

We found that Tfh2 and Tfh17 cells are involved in the occurrence and development of RA to a certain extent. Participating in the inflammatory response and joint destruction process of RA, IL-6, IL-17, TGF-β and MMP-3 can also reflect RA disease activity. The expression levels of Tfh2 and Tfh17 cells are positively correlated with serum IL-6, IL-17 and MMP-3 concentrations, negatively correlated with serum TGF-β concentration. Tfh2 and Tfh17 are expected to be new targets for immunotherapy in RA patients.

Conflict of Interest
This study was supported by Sichuan Province Health Department (16PJ102), Natural Science Foundation of China (Grant No. 31701104), Natural Science Foundation of Chengdu Medical College (CYZ15-10), Sichuan Province's Office of Education (16ZA0289) and National Undergraduate Innovation and Entrepreneurship Program(201513705007).

Conflict of Interest
The Authors declare that they have no conflict of interests.

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