CD4+CXCR5+PD-1+ T Follicular Helper Cells Play a Pivotal Role in the Development of Rheumatoid Arthritis

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
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Background: T follicular helper (Tfh) cells are a subgroup of activated CD4+ T cells in the germinal centers of secondary lymphoid organs, they play critical roles in the development of many chronic autoimmune inflammatory diseases. The aim of this study was to investigate whether circulating Tfh cells contribute to the development of rheumatoid arthritis (RA).

Material/Methods: Thirty patients fulfilled the diagnosis criteria that was established by the American College of Rheumatology and 30 healthy controls were recruited. The frequency of Tfh cells in patients and collagen-induced arthritis (CIA) in DBA/1J mice were analyzed by flow cytometry. The serum IL-21 level was examined by enzyme-linked immunosorbent assay (ELISA). The mRNA expression of Blimp-1 and Bcl-6 were detected by qRT-PCR.

Results: RA patients had more CD4+PD-1+CXCR5+ Tfh cells in peripheral blood compared with healthy controls, and CIA in DBA/1J mice showed similar results. Higher mRNA expression of Bcl-6 and lower Blimp-1 mRNA expression were observed in patients with RA compared to healthy controls, and the expression level of IL-21 was higher in RA patients, which was also seen in CIA mice. Furthermore, the spleen CD4+ICOS+CXCR5+ Tfh cells in CIA mice show significantly higher frequency than that in the control mice. The percentage of CD4+PD-1+CXCR5+ Tfh cells was correlated positively with the values of erythrocyte sedimentation rate (ESR) (r=0.968, $P<0.001$), rheumatoid factor (RF) (r=0.962, $P<0.001$), C-reactive protein (CRP) (r=0.953, $P<0.001$), and anti-cyclic citrullinated peptide antibodies (ACPA) (r=0.966, $P<0.001$), and the level of serum interleukin (IL)-21 in RA patients showed positive correlation with ESR (r=0.982, $P<0.001$), RF (r=0.959, $P<0.001$), CRP (r=0.951, $P<0.001$), and ACPA (r=0.971, $P<0.001$) as well.

Conclusions: The activated Tfh cells in the peripheral blood may be responsible for the development of RA.

MeSH Keywords: **Arthritis, Experimental • Proto-Oncogene Proteins c-bcl-6 • Receptors, Interleukin-21 • Rheumatic Fever • T-Lymphocytes, Helper-Inducer**

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Background

Rheumatoid arthritis (RA) is a systemic autoimmune disease accompanied by hyperplastic synovium, cartilage degradation, and bone destruction [1,2]. It is identified by the presence of circulating auto-antibodies including anti-cyclic citrullinated peptide antibodies (ACPA) and rheumatoid factor (RF) [3,4]. Immunocompetent cells including B cells, T cells, dendritic cells, neutrophils, and natural killer cells, as well as soluble factors such as chemokines and cytokines, are thought to contribute to disease pathogenesis [5,6]. Studies have demonstrated that CD4+ T cells and their secreted cytokines are important in the induction and aggravation of the inflammation [7,8].

Follicular helper T (Tfh) cells are a subgroup of CD4+ T cells that are localized in the B-cell follicle [9,10]. With the help of CD4+ T cells, activated B cells migrate into lymphoid follicles of lymphoid organs and generate germinal centers, differentiating into plasma cells that secrete RF, ACPA, and other autoreactive antibodies [11,12]. Tfh cells are defined by the expression of transcription factor B-cell lymphoma 6 (BCL-6), surface molecules including CD40 ligand, chemokine (C-X-C) receptor 5 (CXCR5), programmed cell death protein-1 (PD-1), inducible T-cell co-stimulator (ICOS), and cytokines such as interleukin (IL)-21, IL-6 and IL-10 [13,14]. Blimp-1 is a transcription factor that acts as an antagonist of BCL-6. The fate of Tfh cell differentiation is dependent on the balance between BCL-6 and Blimp-1 in T cells [15]. It is well-known that uncontrolled generation of Tfh cells in the germinal centers or peripherals could contribute to autoimmunity diseases such as systemic lupus erythematosus and type 1 diabetes, however, the potential role of Tfh cells in the progress of rheumatoid arthritis is not well known [16–18].

In the present study, we investigated whether circulating Tfh cells contribute to the development of RA. We found increased Tfh cells in the peripheral blood of RA patients and collagen-induced arthritis (CIA) mice, and there are close correlations between the increase of Tfh cells, and IL-21, serum C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), RF, and ACPA in RA patients.

Material and Methods

Chemicals

Complete Freund's adjuvant together with immunization grade bovine type II collagen solution were purchased from Chondrex, Inc. (Redmond, WA, USA). Enzyme-linked immunosorbent assay (ELISA) kit for the detection of IL-21 (ml058065) was bought from Shanghai Enzyme-linked Biotechnology Co., Ltd. (Shanghai, China). The primers were synthesized by Sangon Biotech Co.,

Table 1. Clinical characteristics and peripheral blood values of study participants: rheumatoid arthritis patients and healthy controls.

Characteristics	Patients		Healthy controls	
n	30		30	
Age (years)	49	(28–71)	45	(26–72)
Female, n (%)	25	(83%)	12	(40%)
ESR (mm/hour)	41	(10–97)	9	(3–18)
CRP (mg/L)	20.43	(0.73–77.8)	3.1	(0.2–7.6)
ACPA (RU/mL)	102	(8.38–300)	2.6	(0–4.3)
RF (IU/mL)	92.6	(10.6–267.2)	10.4	(0.4–18.2)

ESR – erythrocyte sedimentation rate; CRP – C-reactive protein; ACPA – anti-cyclic citrullinated peptide antibodies; RF – rheumatoid factor.

Ltd. (Shanghai, China). The reverse transcription (RT) kit was supplied by Promega Corporation (Madison, WI, USA), the SYBR Green PCR Master Mix, bicinchoninic acid (BCA) and enhanced chemiluminescence (ECL) kits were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

Patients

There were 30 patients with new-onset RA (<6 months of disease duration) in accordance with the diagnosis criteria established by the American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) [19], who were admitted by the Rheumatology Department of the General Hospital of Ningxia Medical University from November 2016 to February 2018. Thirty healthy controls with matched gender and age were recruited from the same hospital. RA patients were not included in the study if they received treatment of steroids, biological disease-modifying antirheumatic drugs, methotrexate, sulfasalazine, or leflunomide in the past 6 months, or if they had other chronic inflammatory and autoimmune diseases. The collection of peripheral blood of health controls and RA patients was approved by the Ethics Committee of the Ningxia Medical University (No.2015-111). Description of the patients are shown in Table 1.

Animals

Male DBA/1J mice (18–22 g) were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Permit Number: 201708981). They had free access to food and water, and they were housed in multilayer laminar flow racks under a controlled environment (20–25°C and 12-hour light: dark cycle). This study was conducted according to the "Guiding Principles for the Care and Use of Laboratory Animals" [20] and all programs by the Animal Care and Use Committee of Ningxia Medical University.

Establishment of CIA

We used 0.1 mL of bovine type II collagen in an emulsion with complete Freund's adjuvant (1: 1, v/v) to inject subcutaneously into the tail (at approximately 2 cm from the base of the tail) of male DBA/1J mice (8 weeks old) mice [21]. After 21 days, 0.1 mL of collagen emulsion in the same concentrations as the first immunization, substituting CFA for IFA in incomplete Freund's adjuvant (1: 1, v/v) were injected into the tail of the mouse closer to the base of the tail than the original injection site. The control mice were treated in the same way without the CII antigen. The progression of CIA in DBA/1J mice were assessed every 3 days between day 1 and day 45. Erythema, swelling, and scleroma were examined and graded in each paw, and the 4 scores were summed so that the maximum possible score was 16 per mouse. The score was calculated at 5-point scale [8]: 0 was normal, no redness and inflammation; 1 was swelling and redness in one digit; 2 was signs involving the ankle plus tarsal of the hind paw and/or wrist plus carpals of the fore paw; 3 was swelling and redness in all digits and joints; 4 was severe disease involving the entire hind or fore paw.

Histological analysis of knee joints

On day 45 after the first injection, DBA/1J mice were sacrificed after anesthesia and serum samples were collected. Knee joints were fixed in 10% neutral buffered formalin solution for 24 hours and then decalcified in 10% ethylene diamine tetraacetate (EDTA) for 30 days with the EDTA solution renewed once a week, followed by embedding in paraffin. 3-μm thick sections were stained with hematoxylin and eosin (H&E).

Biochemical and immunological measurements

Peripheral venous blood samples involved K2-EDTA blood samples were obtained from individual participants, the values of ESR, the serum levels of CRP, RF, and ACPA were measured by scatter turbidimetry (Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany) in the clinical laboratory of the General Hospital of Ningxia Medical University.

Measurement of serum IL-21

IL-21 in RA patients and healthy controls serum was determined by ELISA according to the manufacturer's instructions. To measure IL-21 in mice, DBA/1J mice were sacrificed on the final day of experimentation and then the serum was collected. The absorbance at 450 nm was then detected by an ELISA reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and plotted against a standard curve with standard levels expressed as pg/mL. Each sample was examined in triplicate.

Spleen lymphocyte preparation

DBA/1J mice spleens were placed in phosphate-buffered saline (PBS) immediately after sacrifice and mechanically disrupted through a wire mesh strainer with the end of a 10 mL plastic syringe plunger. Cell debris were removed from cell suspensions by filter through lens tissue. After centrifugation at 800 rpm/minute for 5 minutes at 4°C, cells were resuspended and dispersed in trypsin followed counting using a hemocytometer.

RNA purification and real-time quantitative PCR analysis

Total RNA of peripheral blood mononuclear cells (PBMCs) was purified by TRIzol. Reverse transcription kit was used to synthesize complementary DNA (cDNA). Real-time quantitative PCR was performed as following: 95°C for 2 minutes, 40 cycles at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 45 seconds. Primers used for the PCR were: BCL-6: sense, 5'-ACCAGAATGCCAAACCAGAG-3', and antisense, 5'-TCAAGGTTCTCAGGCTCCAT-3'; Blimp-1: sense, 5'-AGCAGCGACGAAGCCATGAATC-3' and antisense, 5'-TGCACTCTCAGGTGGACCTTCAG-3'. The relative mRNA expression was calculated using the $2^{-\Delta\Delta Cq}$ method [22], and normalized using β-actin as the reference gene.

Flow cytometry analysis

Blood was density-gradient centrifugated using Ficoll-Paque Plus (Amersham Biosciences, Little Chalfont, UK) to isolate PBMCs. PBMCs at 2×10^6/tube were stained with Percpcy5.5 anti-human CD4 (Cat. No. 560650), AF488 anti-human CXCR5 (Cat. No. 558112), and APC anti-human PD-1 (Cat. No. 558694) (BD Bioscience, San Diego, CA, USA). PBMCs from DBA/1J mice at 2×10^6/tube were stained with PE anti-mouse ICOS (Cat. No. 552146), FITC anti-mouse CXCR5 (Cat. No. 560577) and Percpcy5.5 anti-mouse CD4 (Cat. No. 550954) (BD Bioscience, San Diego, CA, USA) for 30 minutes. After washing with PBS, the cells were analyzed on a BD fluorescence activated cell sorter (FACS) Accuri C6.

Statistical analyses

Data were analyzed using the mean ± standard deviation. Statistical significance was calculated by Student's t-test, one-way analysis of variance (ANOVA), or Pearson's correlation coefficient. A values of $P<0.05$ was considered as statistically significant difference. All statistical analyses were performed using SPSS version 17.0 software (SPSS, Inc., Chicago, IL, USA).
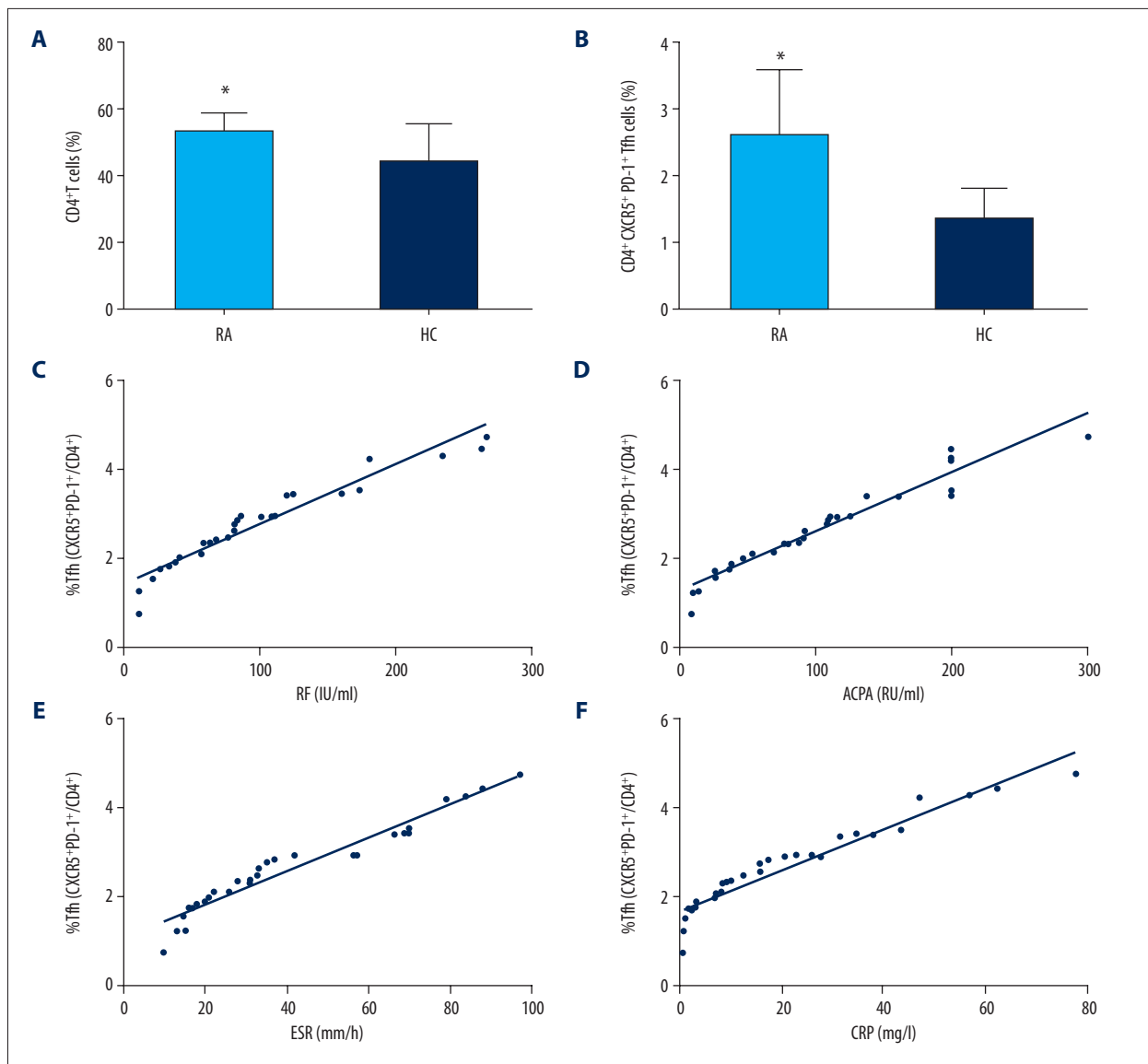
Figure 1. Frequency of circulating follicular helper T (Tfh) cells was increased and associated positively with the levels of autoantibodies and inflammation markers in new-onset rheumatoid arthritis (RA) patients. Peripheral blood mononuclear cells (PBMCs) from RA patients and healthy controls were stained with labelled antibodies as previously described, and analyzed by flow cytometry. (**A**) Percentage of CD4+ T lymphocytes in RA patients and healthy controls. (**B**) Percentage of CD4+ CXCR5+ PD-1+ T cells in RA patients and healthy controls (* *P*<0.01). (**C**) The percentage of Tfh cells was correlated positively with the level of serum RF (rheumatoid factor) in new-onset RA (r=0.962, *P*<0.0001). (**D**) The percentage of Tfh cells was correlated positively with the level of serum ACPA (anti-cyclic citrullinated peptide antibodies) in new-onset RA (r=0.966, *P*<0.0001). (**E**) The percentage of Tfh cells was correlated positively with the value of ESR (erythrocyte sedimentation rate) in new-onset RA (r=0.968, *P*<0.0001). (**F**) The percentage of Tfh cells was correlated positively with the level of serum CRP (C-reactive protein) in new-onset RA (r=0.953, *P*<0.0001).

Results

Circulating Tfh cells increased in new-onset RA patients

To know whether circulating Tfh cells play a role in the development of RA, 30 new-onset RA patients and 30 gender- and age- matched healthy controls were recruited. By ELISA analysis, we found significant higher levels of serum RF, ACPA, CRP, and the values of ESR in the RA patients compared to the healthy controls, meaning that inflammation and autoantibodies attributed to the pathological progression in RA. Among effector CD4+ cells, Tfh cells are critical for B cell activation and they contribute to autoimmunity development. The chemokine receptor CXCR5 enables the Tfh cells have access to the follicles,
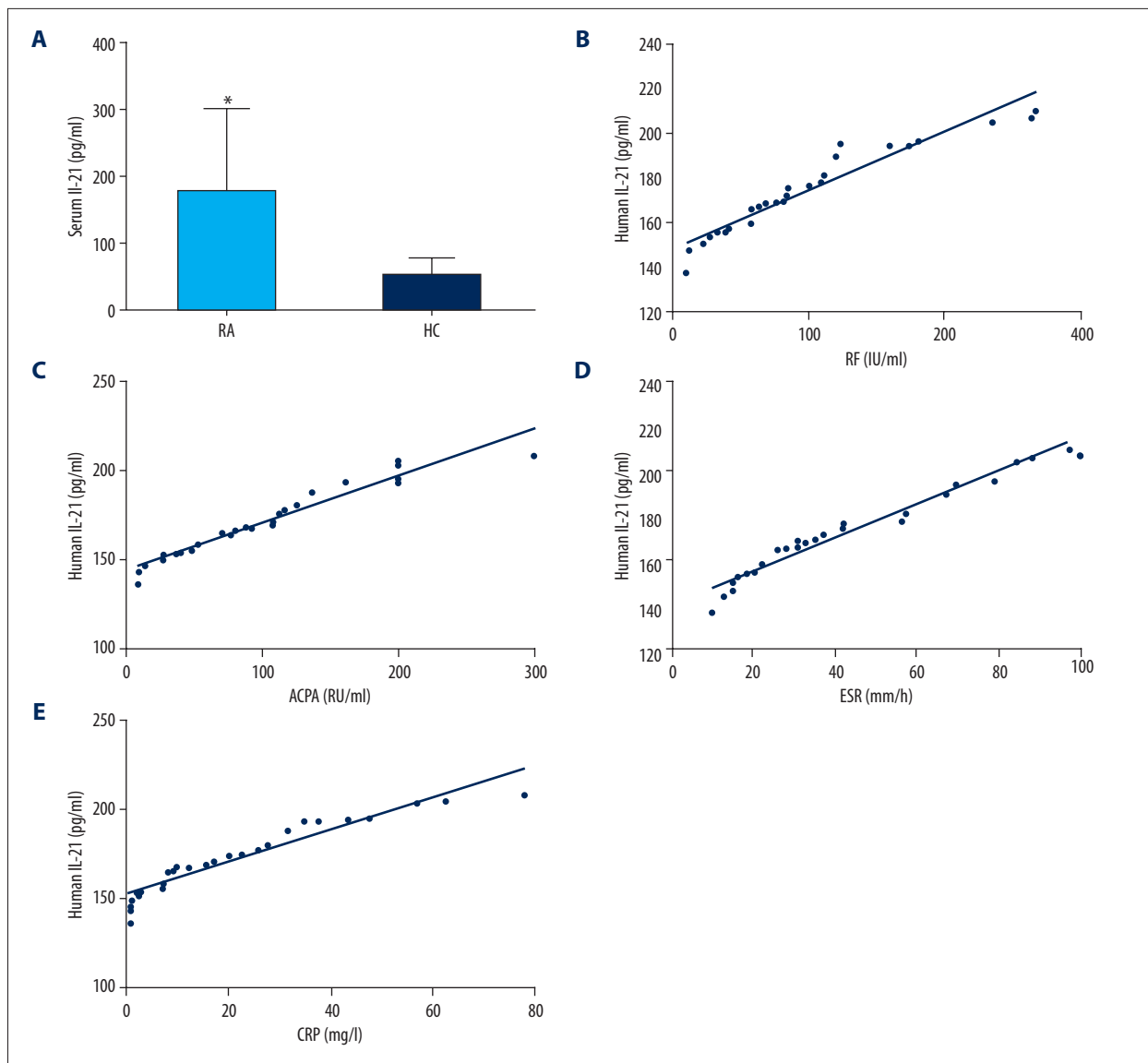
Figure 2. Elevated interleukin (IL)-21 concentration in new-onset rheumatoid arthritis (RA) patients. (**A**) IL-21 expression by enzyme-linked immunosorbent assay (ELISA), * $P<0.05$ compared with healthy controls. (**B**) The level of IL-21 was correlated positively with the level of serum RF (rheumatoid factor) in new-onset RA ($r=0.971$, $P<0.0001$). (**C**) The level of IL-21 was correlated positively with the level of serum ACPA (anti-cyclic citrullinated peptide antibodies) in new-onset RA ($r=0.959$, $P<0.0001$). (**D**) The level of IL-21 was correlated positively with the value of ESR (erythrocyte sedimentation rate) in new-onset RA ($r=0.982$, $P<0.0001$). (**E**) The level of IL-21 was correlated positively with the level of serum CRP (C-reactive protein) in new-onset RA ($r=0.951$, $P<0.0001$).

programmed cell death protein-1 (PD-1) deliver survival signals to germinal center-B cells. In order to know the possible role of Tfh cells in the progress of RA, the frequency of peripheral blood CD4$^+$ CXCR5$^+$ PD-1$^+$ Tfh cells in total CD4$^+$ T cells were characterized by flow cytometry analysis. The results showed significantly higher percentage of CD4$^+$ CXCR5$^+$ PD-1$^+$ Tfh cells in the RA patients than in the healthy controls (Figure 1A, 1B), suggesting that higher frequency of the circulating Tfh cells might be responsible for the disease development of RA.

Increased circulating Tfh cells were correlated with high levels of autoantibodies and inflammation markers in patients with new-onset RA

In order to know whether the circulating Tfh cells were associated with the plasma auto-antibodies such as ACPA and RF, the correlation between the levels of auto-antibodies and the frequency of the circulating Tfh in RA patients was examined. The frequency of the circulating Tfh cells showed positive
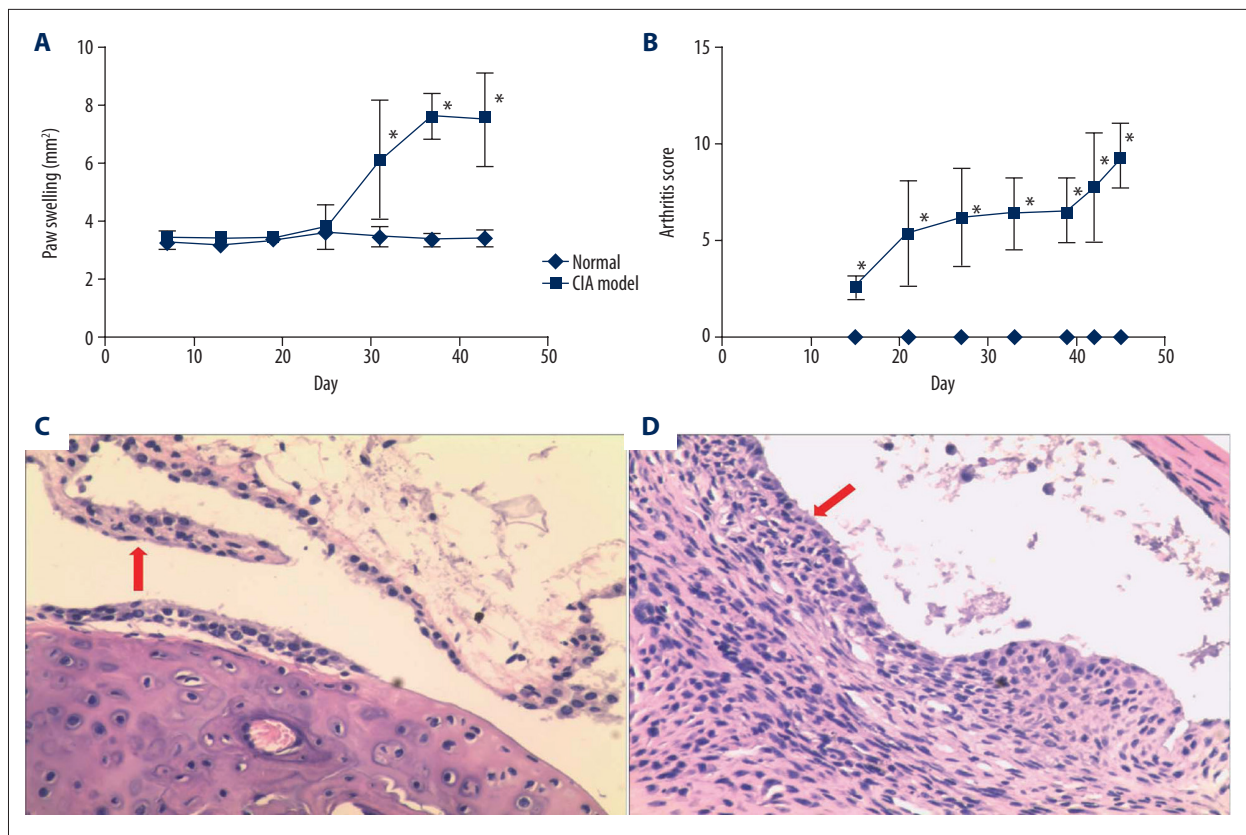
Figure 3. Assessment of collagen-induced arthritis in DBA/1J mice. (**A**) Paw swelling (mean ± standard deviation (SD), n=10 per group) were significant increase in collagen-induced arthritis (CIA) model. (**B**) Arthritic scores (mean ±SD, n=10 per group) were significant increase in CIA model. (**C**) Hematoxylin and eosin (H&E) staining of paw sections in normal mice (10×20, arrow). (**D**) H&E staining of paw sections in CIA model mice (10×20, arrow). Histological joint destruction in paw sections revealed remarkable synovial hyperplasia, inflammatory cell infiltration into the joint capacity in CIA mice compared with the normal mice. * $P<0.05$, compared with the normal mice.

correlation with RF (r=0.962, $P<0.0001$) or ACPA (r=0.966, $P<0.0001$) (Figure 1C, 1D) and inflammation markers ESR (r=0.968, $P<0.0001$) or CRP (r=0.953, $P<0.0001$) in new-onset RA patients (Figure 1E, 1F). These findings indicated that Tfh cells might be the reason of increased production of pathogenic auto-antibodies and inflammation.

Increased Tfh cells lead to increased circulating IL-21 in RA patients and CIA mice

IL-21 is a signature cytokine of Tfh cells; it can regulate B cell differentiation and proliferation, and enhance B cells to produce antibodies. We found that the concentration of IL-21 in the serum of new-onset RA patients was significantly higher than those of healthy controls ($P<0.01$) (Figure 2A). Additionally, the IL-21 concentration was related to the production of autoantibodies RF (r=0.971, $P<0.0001$) or ACPA (r=0.959, $P<0.0001$) (Figure 2B, 2C) and inflammation markers ESR (r=0.982, $P<0.0001$) or CRP (r=0.951, $P<0.0001$) in RA patients (Figure 2D, 2E). To further prove our results,

the collagen-induced arthritis in DBA/1J mice model was established. As shown in Figure 3A and 3B, there were a significant increase in hind paw volume and the arthritic scores in the CIA mice group compared to the normal control ($P<0.05$) (Figure 3A, 3B). Histopathological examination of the knee joints by H&E staining revealed remarkable synovial hyperplasia, inflammatory cell infiltration into the joint capacity of CIA mice compared with the normal control mice (Figure 3C, 3D). Flow cytometry analysis showed that the percentage of CD4+ CXCR5+ ICOS+ Tfh cells in CIA mice were significantly higher than those in the control mice (Figure 4A), and the level of IL-21 in the serum of CIA mice was higher than those of the control mice ($P<0.01$) (Figure 4B), suggesting that increased frequency of activated Tfh cells in the RA mouse model, which further supports our results from RA patients. These data indicate that IL-21 produced by Tfh cells might contribute to the development of RA.
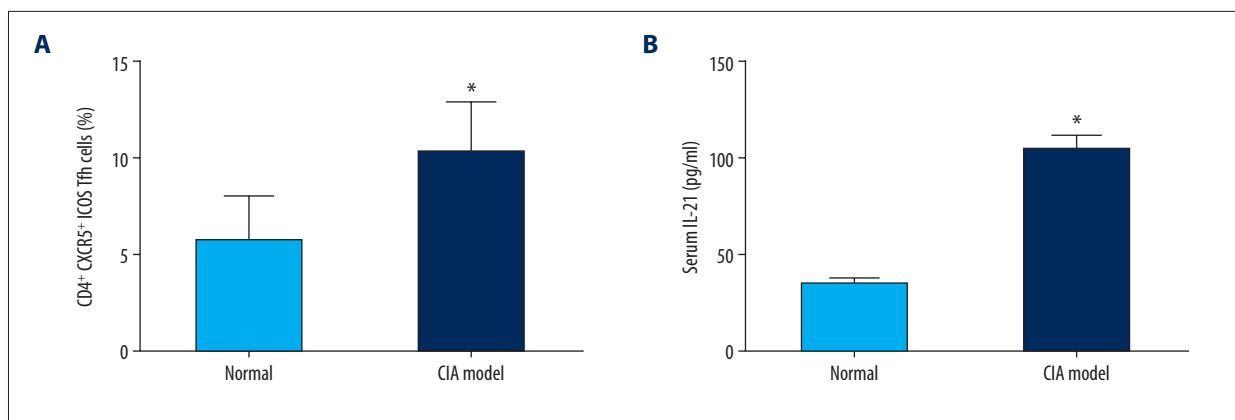
Figure 4. Increased frequency of the circulating Tfh cells and high serum concentration of interleukin (IL)-21 in collagen-induced arthritis (CIA) mice. (**A**) Percentage of CD4+ CXCR5+ ICOS+ T cells in CIA model mice. (**B**) Serum concentration of IL-21 in CIA mice. * P<0.05, compared with the normal mice.
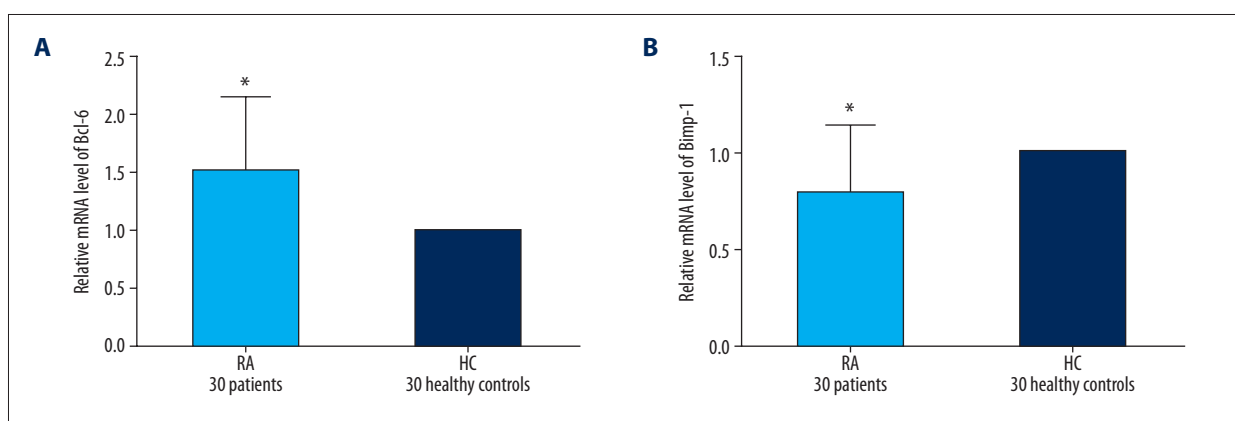

Figure 5. Decreased Blimp-1 mRNA expression and elevated Bcl-6 mRNA expression lead to increased Tfh cells in new-onset rheumatoid arthritis patients. (**A**) Bcl-6 mRNA expression by qRT-PCR. (**B**) Blimp-1 mRNA expression by qRT-PCR. * P<0.05 compared with healthy controls.

Decreased expression of Blimp-1 and elevated expression of Bcl-6 lead to increased Tfh cells in RA Patients

B-cell lymphoma 6 (BCL-6) is an important transcription factor in Tfh cells, and Blimp-1 acts as an antagonist of BCL-6. The balance between BCL-6 and Blimp-1 plays a critical role in Tfh cell differentiation. To further examine whether Tfh cells in RA patients are important for RA pathogenesis, the mRNA expression of Bcl-6 and Blimp-1 in RA patients and healthy controls were examined by qRT-PCR. The results showed that Bcl-6 mRNA expression of RA patients was higher than that in healthy controls (Figure 5A) while the level of Blimp-1 mRNA was decreased in RA patients (Figure 5B).

Discussion

Rheumatoid arthritis is an autoimmune disease which is characterized by autoantibodies produced by B cells, such as RF

and ACPAs. It is known that the production of antibodies is caused by interaction between T-B cells. Tfh cells are a novel subpopulation of CD4+ T cells that are specialized in the germinal centers of secondary lymphoid organs, where they promoted B cell functional differentiation and maturation and sustained antibody production in the periphery [23,24]. Data have shown that the number of CD4+ ICOS+ T cells increased in the peripheral blood of patients with systemic lupus erythematosus (SLE) and myasthenia gravis (MG) [25,26]. In this study, we found significant increased frequency of CD4+ CXCR5+ PD-1+ circulating Tfh cells in the peripheral blood of RA patients. Tfh cell numbers are important for germinal center B cell survival and affinity maturation, however, excessive Tfh cells have the potential to drive aberrant germinal center B-cell survival, possibly due to the generation of autoantibodies to establish autoimmune disease.

Bcl-6 is an essential transcription factor for Tfh cells; it regulates Tfh cells differentiation and maturation [27]. Blimp-1 is

an antagonist of Bcl-6, it limits the differentiation of T cells into Tfh cells. The expression of Bcl-6 is dependent on IL-6 and IL-21; it can identify Tfh cells from other polarized T helper cell subsets as well [28,29]. IL-21 is the hallmark cytokine of Tfh cells and it acts as a potent inducer of plasma cell and induces the secretion of IgG, IgM, and IgA [30]. Some previous studies have demonstrated that increased frequency of Tfh cells correlated with higher levels of serum IL-21 in RA patients compared with healthy controls, and the increased concentration of IL-21 could promoted the mRNA expression of Bcl-6 [31,32]. In this study, we found upregulated Bcl-6 mRNA expression and downregulated Blimp-1 mRNA expression in RA patients, indicating an increase in T cell differentiation into Tfh cells in RA patients. Our results revealed significant high serum IL-21 in the RA patients. Moreover, the serum concentration of RF, ACPA, ESR, and CRP was also associated with the IL-21 which can be produced by Tfh cells. These data suggested that Tfh cells might contribute to the pathogenesis of RA.

It has been noticed that the increase of Tfh cells does not always coincide with increased levels of auto-antibodies such as ACPAs and RF in serum. Recent studies demonstrated that CD4^{+} CXCR5^{+} Tfh cells could be differentiated into CXCR3^{+} CCR6^{−} Tfh1, CXCR3^{−}CCR6^{−} Tfh2 and CXCR3^{−}CCR6^{+} Tfh17 subsets; comparing to the RA patients to the healthy controls in former study found no difference observed in the Tfh1 or in Tfh17 subset, and no relationship was found between the frequencies of CD4^{+} CXCR5^{+} Tfh cells and anti-MCV antibodies or RF, ESR, or CRP [33]. The observation from Ma et al. [32] also showed that there was a positive correlation between anti-CCP and the circulating Tfh cells, and no relationship with RF. In our study, we found a positive correlation between CD4^{+}CXCR5^{+}PD-1^{+} Tfh cells and auto-antibodies. In addition, we observed a positive correlation between CD4^{+}CXCR5^{+}PD-1^{+} Tfh cells and ESR and CRP. It is known that RA is a chronic and systematic autoimmune inflammatory disease, and ESR and CRP reflect the degree of inflammation in the blood. Our study proved that the frequency of Tfh cells increased in the RA patients and was positively correlated with serum anti-bodies and inflammatory markers.

To better understand the role of Tfh cells in the development and progression of RA, we established collagen-induced arthritis (CIA) in DBA/1J mice to further study RA. We found the possible contribution of Tfh cells to the pathogenesis of RA by examination of the percentage of CD4^{+} CXCR5^{+} ICOS^{+} Tfh cells in the spleen and the level of IL-21 in CIA mouse serum. Recent studies support that IL-21 can be secreted by Tfh cells and it can serve as a key regulator of humoral responses by direct regulation of B-cell proliferation and class switching.

Conclusions

Our research data suggested that the circulating Tfh cells were involved in the pathological progression of RA and the percentage of circulating Tfh cells was a valuable biomarker of the uncontrolled responses of antibodies in RA. However, the activation mechanisms of Tfh cells, and how IL-21 regulates B cell and Tfh cell activation and differentiation in RA patients, needs to be further explored in the future.

Acknowledgements

Conflicts of interest

None.

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