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ORIGINAL ARTICLE

Imbalance of circulating Tfr/Tfh ratio in patients with rheumatoid arthritis

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Abstract

Follicular helper T(Tfh) cells and follicular regulatory T(Tfr) cells are critical for the development and maintenance of germinal center and humoral immune responses. Accumulating evidence has demonstrated that the dysregulation of either Tfh or Tfr cells contributes to the pathogenesis of autoimmune diseases. The aim of this study was to examine the numbers of Tfh and Tfr cells in patients with rheumatoid arthritis (RA). Twenty-four patients with RA patients and 20 health controls (HCs) were enrolled in this study. We analyzed the numbers of Tfh ($CD4^+$ $CXCR5^+$ $PD\text{-}1^{hi}$) cells and Tfr ($CD4^+$ $CXCR5^+CD127^{lo}$) cells in 24 RA patients via flow cytometry. The level of the soluble PD-1 and its ligands (sPD-L1 and sPDL-2) were examined by ELISA. Flow cytometry revealed that both circulating Tfh and Tfr cells were increased in RA patients compared with HCs. More importantly, the ratio of Tfr/Tfh was decreased, indicating a disruption of the balance between Tfh and Tfr. The Tfr/Tfh ratio was inversely correlated with level of serum CRP, ESR, RF, anti-CCP, IgG and DAS28 index. We also found that the serum level of sPD-1 was significantly elevated in the RA patients, which was positively correlated with CRP, ESR and the number of Tfh cells. These results indicate that an imbalance of circulating Tfr and Tfh cells may be involved in the immunopathogenesis of RA and may provide novel insight for the development of RA therapies.

Keywords Rheumatoid arthritis · Follicular helper T cell(Tfh) · Follicular regulatory T cell(Tfr) · Tfr/Tfh · PD-1

Abbreviations

RA	Rheumatoid arthritis
cTfh	Circulating follicular helper T
cTfr	Circulating follicular regulatory T
GC	Germinal center
ELISA	Enzyme-linked immunosorbent assay
CRP	C-reaction protein
ESR	Erythrocyte sedimentation rate
IgG	Immunoglobulin G
RF	Rheumatoid factor
CXCR5	CXC chemokine receptor 5
CCP	Cyclic citrullinated peptide
sPD-1	Soluble PD-1
sPD-L1	Soluble PD-L1
sPD-L2	Soluble PD-L2
DAS28	Disease activity score in 28 joints
DMARDs	Disease-modifying antirheumatic drug

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Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by synovial inflammation, cartilage lesions and bone destruction. Many types of immunocompetent cells, such as dendritic cells, macrophages and T and B cells, have been found to contribute the pathogenesis of RA [1–3]. Among these, adaptive immunity mediated by autoreactive T cells plays a central role through stimulating B cells and macrophages to produce autoantibodies and proinflammatory cytokines.

Indeed, various T cell subsets and molecules linked to T cell functions are suggested to be involved in the pathogenesis of autoimmune disease. T follicular helper cells (Tfh) cells, a T helper (Th) cell subset, are essential for the

selection of high-affinity B cells, the germinal center (GC), and can promote antibody production [4, 5]. In contrast, follicular regulatory T (Tfr) cells were recently defined as a specialized population of Tregs which suppress the GC reaction after immunization and thus play an opposing role with Tfh cells in the regulation of humoral immunity. Accumulating evidence has demonstrated that abnormal either Tfr or Tfh cells activity may result in the dysregulation of immune tolerance and abnormal production of high levels of autoantibodies and thereby contribute to the pathogenesis of autoimmune responses [6–9].

Classical Tfh and Tfr cells are primarily located in secondary lymphoid organs, which prevent their routine study in human patients [8, 10]. Several reports have subsequently described circulating populations of $CD4^+$ T cells that express CXCR5 and display both phenotypic and functional features of true Tfh and Tfr cells [11–14]. An altered balance of circulating Tfh (cTfh) and Tfr (cTfr) cell counterparts (cTfh and cTfr) has been associated with autoimmune diseases such as systemic lupus erythematosus [15], myasthenia gravis [16], ulcerative colitis [17] and primary biliary cholangitis [18]. To date, though a few articles have focused on the frequency cTfh cell subsets in RA [19–23], the balance of cTfh/cTfr cell and its clinical significance has not been investigated in RA.

In this study, we observed the balance of cTfr/cTfh in RA patients, and the association between the ratio of cTfr/cTfh and important disease markers in RA. Our results suggest that alterations in cTfh and cTfr cells cause a shift from immune tolerance to immune responsive state, contributing to dysregulated immunity and the pathogenesis of RA.

Methods

Blood samples and clinical data

The study was approved by the ethics committee of the first affiliated hospital of China Medical University. Peripheral blood mononuclear cells (PBMCs) were obtained from 24 patients with RA who fulfilled the 2010 American College of Rheumatology/European League Against Rheumatism classification criteria [24], and 20 age- and sex-matched healthy controls (HCs). All subjects provided informed consent according to the Declaration of Helsinki. The enrolled participants were inpatients from the Department of Rheumatology and Immunology of the First Affiliated Hospital of China Medical University during 2016–2017. Some of the RA patients were untreated, and another received intermittently traditional or biological disease-modifying antirheumatic drugs. The disease severity was evaluated by a disease activity score in 28 joints (DAS28) [25]. Clinical characteristics and laboratory data were documented on the day of sample collection, which are summarized in Table 1.

Cell isolation and flow cytometry

Peripheral blood mononuclear cells (PBMCs) were separated from EDTA-anticoagulated whole blood within 2 h, by Ficoll-Paque™ PLUS (Amersham Biosciences Co, Piscataway, NJ, USA) density gradient centrifugation. 1×10^6 PBMCs were re-suspended in RPMI containing 10% fetal bovine serum and stained with antibodies to surface markers at 4 °C for 30 min.

The antibodies included Percp-cy5.5-conjugated anti-CD3 antibody (clone SP34-2; BD Biosciences), FITC-conjugated anti-CD4 antibody (clone RPA-T4; BD Biosciences, San Jose, CA, USA), PE-conjugated anti-PD-1 antibody (clone PD1.3.1.3; MiltenyiBiotec, BergischGladbach, Germany), APC-conjugated anti-CD127 antibody (clone MB15-18C9; MiltenyiBiotec, BergischGladbach, Germany), PE-Vio770-conjugated anti-CXCR5 antibody (clone REA103; MiltenyiBiotec, BergischGladbach, Germany), PE-conjugated anti-CD20 antibody (clone 2H7; Biolegend), APC-conjugated anti-CD19 antibody (clone HIB19; Biolegend) and their isotype controls. After staining, PBMCs were washed twice with FACS buffer and analyzed by FACSAriaTMIIU (BD Biosciences, San Jose, CA). Cells were first gated based on the forward/side scatter followed by gating on cells positive for side scatter and CD4. After collecting bulk $CD4^+$ T cells, the following two populations were collected based on CXCR5 and PD-1 or CXCR5 and

Table 1 Clinical characteristics and laboratory data

Parameter	RA ($n=24$)	HC ($n=20$)
Disease duration (month)	111.8 ± 131.8	–
Age (year)	58.3 ± 11.3	48.2 ± 10.8
Male/female	5/19	4/16
ESR (mm/h)	48.4 ± 28.7	–
CRP (mg/l)	31.3 ± 18.7	–
DAS28-CRP	4.36 ± 1.7	–
RF (±)	21/3	–
RF (IU/ml)	228.7 ± 352.1	–
Anti-CCP (±)	20/4	–
Anti-CCP (IU/ml)	310.9 ± 231.7	–
White cell count ($\times 10^9$/l)	6.11 ± 2.62	–
Lymphocytes count ($\times 10^9$/l)	2.87 ± 1.83	–

Data are presented as mean ± SD; *t* test, Mann–Whitney *U* test and Wilcoxon signed-rank test were used

ESR erythrocyte sedimentation rate, *CRP* C-reactive protein, *DAS28-CRP* disease activity score in 28 joints, *RF* rheumatoid factor, *CCP* cyclic citrullinated peptide

$P < 0.05$ were considered statistically significant

CD127: circulating Tfh ($CD4^+CXCR5^+PD\text{-}1^{hi}$) cells and Tfr ($CD4^+CXCR5^+CD127^{lo}$) cells.

Measurement of serum sPD-1, sPD-L1 and sPD-L2

Serum levels of sPD-1, sPD-L1, sPD-L2 were, respectively, determined by enzyme-linked immunosorbent assay (ELISA) (R&D Systems). All assays were performed according to the manufacturers' protocols.

Statistical analysis

Data are presented as mean ± SEM. Statistical differences between groups were evaluated using an unpaired two-tailed Student's *t* test, the Mann–Whitney *U* test or the Wilcoxon signed-rank test for comparing two groups. Pearson regression was used to assess correlations between variables. *P* values less than 0.05 were considered statistically significant. Calculations were conducted using SPSS 16.0 software (IBM Inc, New York, USA).GraphPad Prism5 (GraphPad Software Inc, CA, USA) was used to create the graphics.

Results

Characteristics of study subjects

A total of 24 patients with RA and 20 gender- and age-matched HC were recruited. The clinic characteristics of the recruited subjects are described in Table 1. There was no significant difference in the distribution of age and gender between the patients and HC. Table 2 reports the treatments in the patients at the time of the FACS analysis.

RA patients demonstrate an altered balance of cTfh subsets, comparing to HC

According to the PD-1 and CD127 expression patterns, peripheral blood $CD4^+CXCR5^+$ cells were classified into cTfh ($CD4^+$ $CXCR5^+PD\text{-}1^{hi}$) cells and cTfr ($CD4^+CXCR5^+CD127^{lo}$) cells. The gating strategies of cTfh and cTfr subsets are shown in Fig. 1. The percentage of $CD4^+CXCR5^+PD\text{-}1^{hi}$ in $CD4^+$ T cells were increased significantly in RA patients significantly compared to HC (4.09 ± 3.04 vs. 0.55 ± 0.51, $P < 0.001$). The percentage of

Table 2 Patients' therapies at the time of the FACS analysis

	MTX	LEF	HCQ	SSZ	MP	TWP	Tofacitinib
Patient 1							
Patient 2					4 mg/qd		
Patient 3							
Patient 4	12.5 mg/qw		200 mg/bid		4 mg/qd	20 mg/tid	
Patient 13							
Patient 5							
Patient 6							
Patient 7							
Patient 8			200 mg/bid				
Patient 9							
Patient 10							
Patient 11							
Patient 12		10 mg/qd	200 mg/bid				
Patient 14	12.5 mg/qw		200 mg/bid		4 mg/qd	20 mg/tid	
Patient 15				750 mg/tid		20 mg/tid	
Patient 16							
Patient 17	12.5 mg/qw						5 mg/bid
Patient 18							
Patient 19		10 mg/qd					
Patient 20	10 mg/qw						
Patient 21	12.5 mg/qw						
Patient 22	12.5 mg/qw		200 mg/bid	750 mg/tid		20 mg/tid	
Patient 23							
Patient 24			200 mg/bid	750 mg/tid	8 mg/qd		

MethotrexateLe (MTX), flunomide (LEF), hydroxylchloroquine sulfate (HCQ), sulfasalazine(SSZ), methylprednisolone (MP). Tripterginum wilfordii polyglycoside (TWP) is a traditional Chinese medicine, which has been used in RA treatment widely

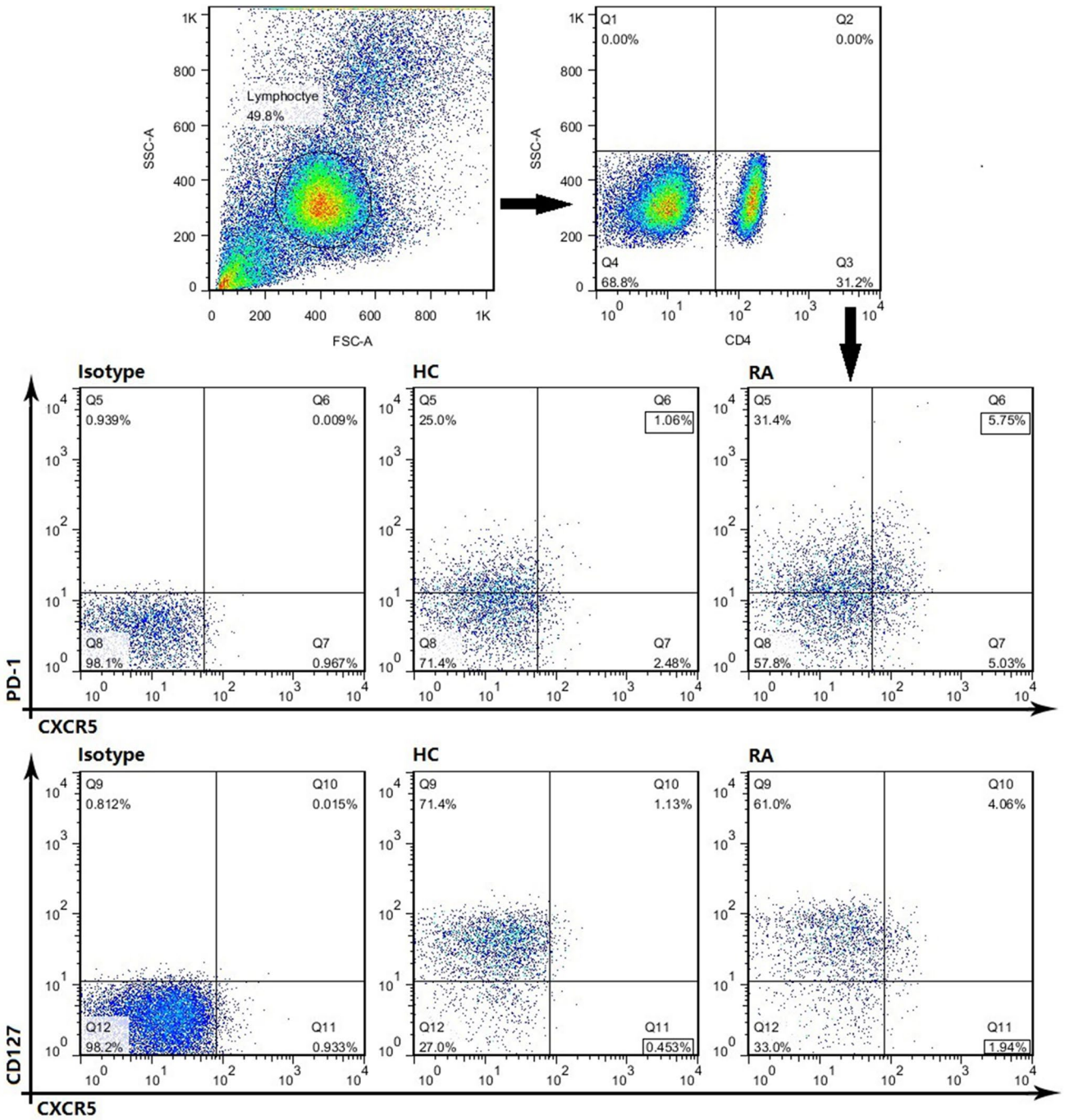

Fig. 1 Flow cytometry analysis of $CD4^+CXCR5^+PD\text{-}1^{hi}$ and $CD4^+CXCR5^+CD127^{lo}$ cells in RA and HC. All of the values were gated on $CD4^+$ T cells. Values in the upper right quadrant of the second row correspond to the percentage of $CD4^+CXCR5^+PD\text{-}1^{hi}$ cells. Values in the lower right quadrant of the third row correspond to the percentage of $CD4^+CXCR5^+CD127^{lo}$ cells

$CD4^+CXCR5^+CD127^{lo}$ in $CD4^+$ T cells were also significantly higher than that in HC (1.93 ± 1.12 vs. 0.50 ± 0.28, $P < 0.001$). The ratio of cTfr/cTfh decreased significantly when compared with HC (0.58 ± 0.35 vs. 1.31 ± 1.15, $P < 0.001$) (Fig. 2).

In our data, we found that the percentages of circulating cTfh ($1.89\% \pm 0.6\%$ vs. $4.99\% \pm 3.18\%$, $P < 0.05$) and cTfr ($1.18 \pm 1.4\%$ vs. $2.24 \pm 1.03\%$, $P < 0.05$) were reduced significantly in MTX-treated group (7 cases) when compared with non MTX-treated group (17 cases) (Fig. 3).

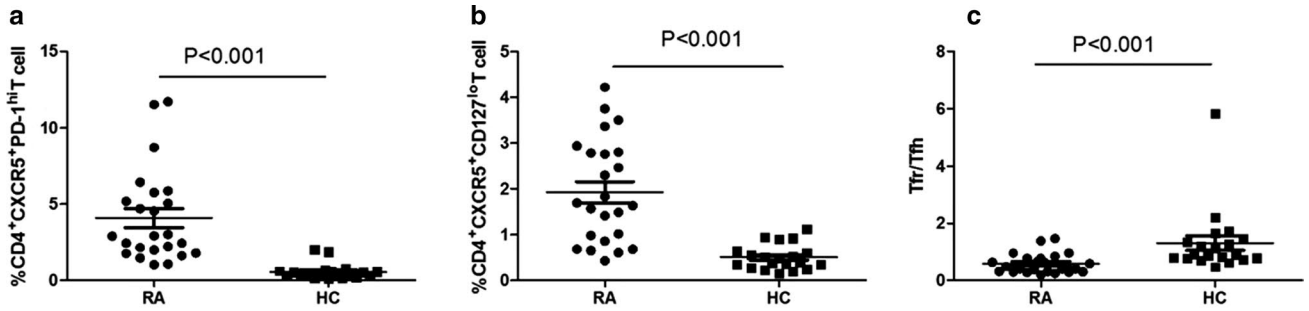

Fig. 2 Percentages of cTfh and cTfr cells in RA patients and HC. The percentage of $CD4^+CXCR5^+PD\text{-}1^{hi}$ in $CD4^+$ T cells were increased significantly in RA patients (**a**); the percentage of $CD4^+$ $CXCR5^+$ $CD127^{lo}$ in $CD4^+$ T cells were increased significantly in RA patients (**b**); the ratio of cTfr/cTfh was decreased significantly in RA patients (**c**). All were compared with HC group

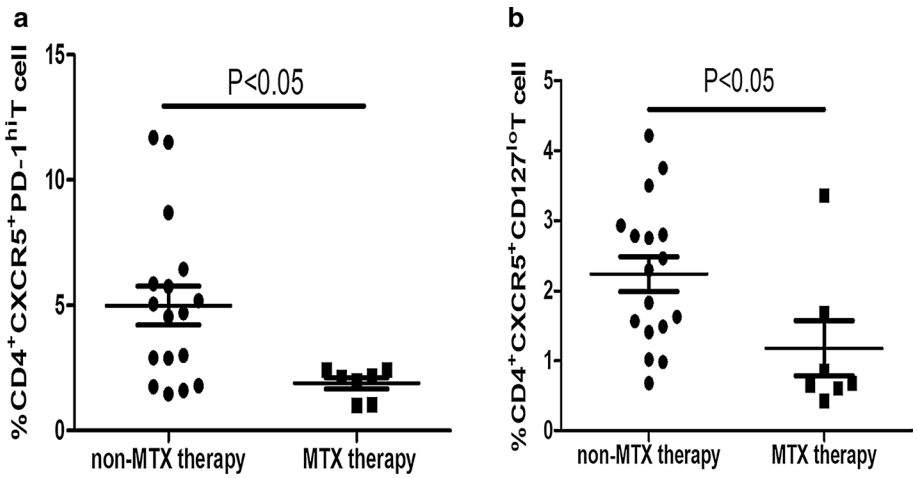

Fig. 3 Differences in circulating Tfh and Tfr among patients with and without methotrexate treatment. The percentages of cTfh (**a**) and cTfr (**b**) in $CD4^+$ T cells were both reduced significantly in MTX-treated group when compared with non-treatment group

Significant increased circulating B cells correlated with Tfh cells in RA patients

To explore the correlation of circulating Tfh and B cells, we examined the circulating $CD3^-CD19^+$ B cells and $CD3^-CD20^+$ B cells in another eight untreated RA patients (Fig. 4). Our results showed that the percentage of $CD3^-CD19^+$ B cells in circulating lymphocytes were significantly higher in RA patients than that in HC (6.3 ± 2.18 vs. 3.86 ± 1.12, $P < 0.05$). $CD3^-CD20^+$ B cells were also increased significantly compared to HC (6.1 ± 2.05 vs. 3.62 ± 1.21, $P < 0.05$) (Fig. 5).

Significant positive correlations were found in the percentage of circulating Tfh cells and $CD3^-CD19^+$ B cells ($r = 0.72$, $P < 0.05$) and $CD3^-CD20^+$ B cells ($r = 0.71$, $P < 0.05$).

The clinical significance of cTfh and cTfr cells in patients with RA

We further determined the potential association between the different types of cells and the clinical data in RA patients. We found that $\%CD4^+CXCR5^+PD\text{-}1^{hi}$ cells positively correlated with the serum levels of CRP, ESR, RF, CCP, IgG and DAS28 index (Table 3a), whereas the ratio of $\%CD4^+CXCR5^+CD127^{lo}/\%CD4^+CXCR5^+PD\text{-}1^{hi}$ negatively correlated with their levels (Table 3b).

Abnormalities of sPD-1, sPD-L1 and sPD-L2 in RA patients

The results of ELISA measurement showed that the serum level of sPD-1 was significantly elevated in the RA patients when compared with HC. There were no significantly differences in the levels of sPD-L1 and sPD-L2 between RA group and HC (Fig. 6). We also found that the serum level of sPD-1 positively correlated with $\%CD4^+CXCR5^+PD\text{-}1^{hi}$cells (Fig. 7), CRP and ESR in RA group (Fig. 8).

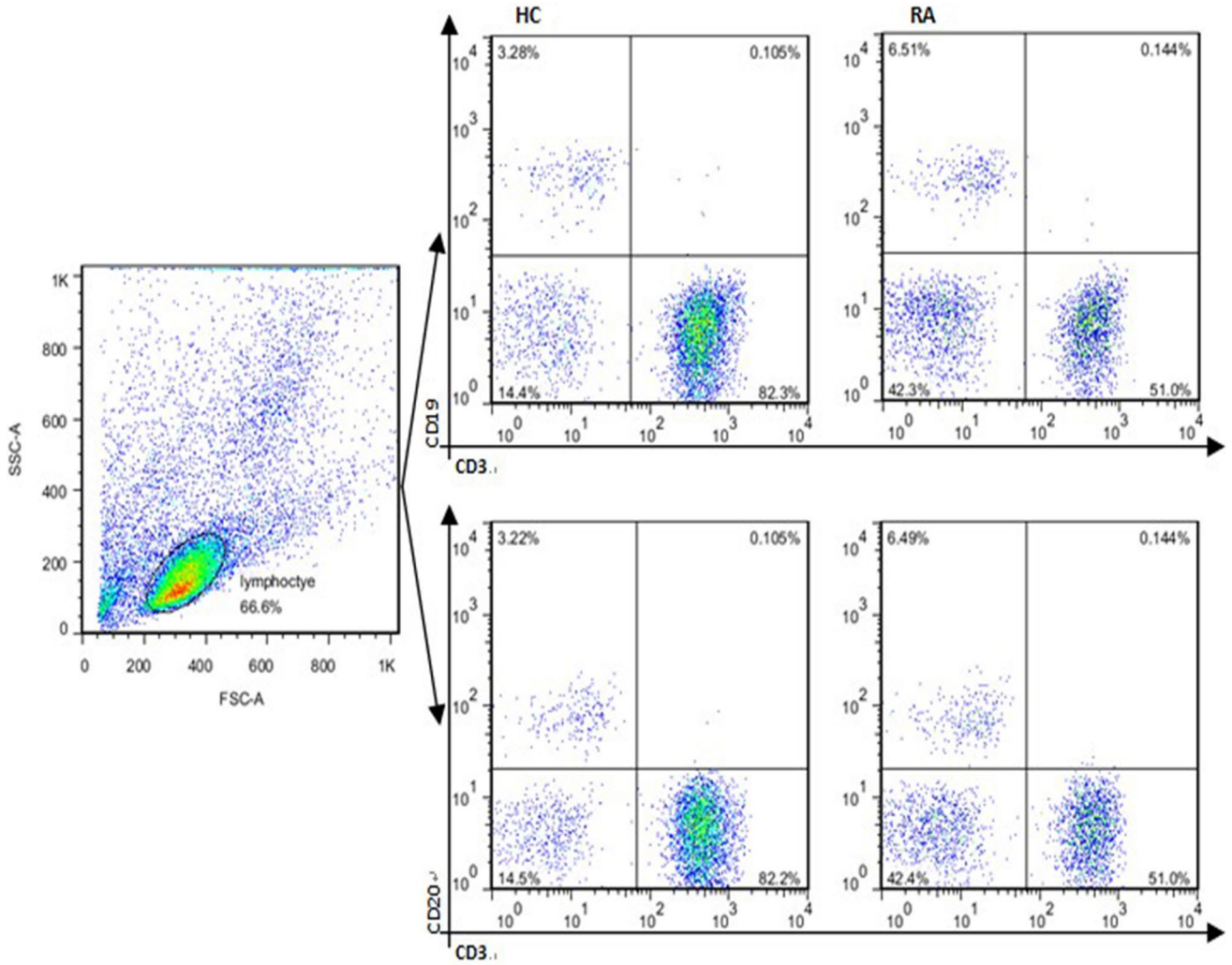

Fig. 4 Flow cytometry analysis of $CD3^{-}CD19^{+}$ B cells and $CD3^{-}CD20^{+}$ B cells in RA and HC. All of the values were gated on lymphocytes. Values in the upper left quadrant of the first row correspond to the percentage of $CD3^{-}CD19^{+}$ B cells. Values in the upper left quadrant of the second row correspond to the percentage of $CD3^{-}CD20^{+}$ B cells

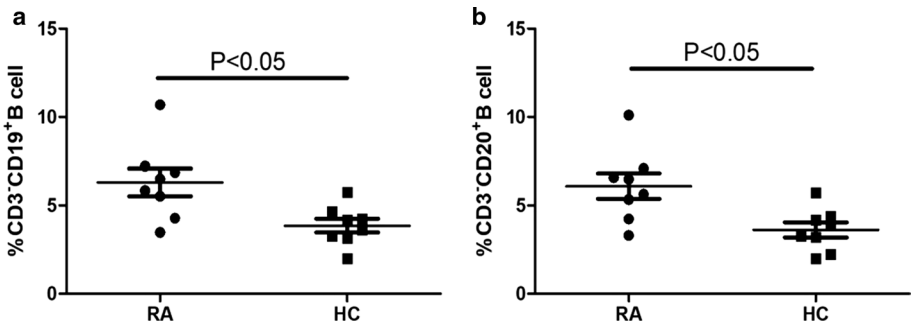

Fig. 5 Percentages of circulating $CD3^{-}CD19^{+}$ B cells and $CD3^{-}CD20^{+}$ B cells in RA patients and HC. The percentage of $CD3^{-}CD19^{+}$ B cells in lymphocytes were increased significantly in RA patients (**a**); the percentage of $CD3^{-}CD20^{+}$ B cells in lymphocytes were increased significantly in RA patients (**b**). All were compared with HC group

Discussion

Tfh cells are important for helping B cell activation and differentiation. Accumulating evidence suggests that Tfh cells are critically involved in the pathogenesis of autoimmune diseases, including RA [4, 7, 19, 23]. CXCR5 and PD-1 are expressed by Tfh cells, and IL-21 is crucial for the development and function of Tfh. In this study, we found that the percentages of circulating Tfh cells were significantly higher in the RA patients than that in the HC. We also demonstrated that there was a significant positive

Table 3 Correlations between (a) percentage of cTfh, (b) ratio of cTfr/cTfh and the clinical parameters in RA group

Parameters	*r*	*p*
(a)		
Disease duration	0.582	**0.003**
CRP	0.470	**0.021**
ESR	0.528	**0.008**
DAS28-CRP	0.452	**0.027**
RF	0.681	**< 0.001**
Anti-CCP	0.522	**0.009**
WBC	−0.353	0.9
Lymphocytes	−0.197	0.356
Hb	−0.198	0.354
PLT	−0.162	0.45
IgG	0.418	**0.042**
IgA	−0.05	0.816
IgM	0.112	0.603
(b)		
Disease duration	−0.109	0.612
CRP	−0.456	**0.025**
ESR	−0.412	**0.048**
DAS28-CRP	−0.519	**0.009**
RF	−0.419	**0.042**
CCP	−0.629	**0.001**
WBC	−0.145	0.5
Lymphocytes	−0.087	0.687
Hb	0.063	0.771
PLT	−0.055	0.799
IgG	−0.41	**0.047**
IgA	−0.109	0.611
IgM	0.016	0.942

Data are presented as mean ± SD. Pearson regression was used to assess correlations between variables

Ly lymphocytes, *Hb* hemoglobin, *PLT* platelet

$P < 0.05$ was considered statistically significant (bold)

correlation between the percentages of circulating Tfh and B cells in RA patients. Our findings extend the previous observations of a higher frequency of Tfh cells in RA patients. Because the number of cTfh cells increased in proportion to their GC counterparts [26], the increase of %cTfh cells may reflect an increased number of activated Tfh cells in the GCs of second lymphoid organs.

PD-1 is expressed on activated T cells, particularly on Tfh cells, and $CD4^+CXCR5^+PD\text{-}1^{hi}$ cells are usually considered to be Tfh cells. PD-1 promotes cognate T–B interactions and delivers inhibitory signals to Tfh cells [5, 9]. Blockade of the interactions between PD-1 and the ligands PD-L1 and/or PD-L2 causes increased Tfh cell differentiation [27], suggesting that PD-1 acts as a negative regulator of cTfh cell differentiation. However, previous reports were inconsistent in the association between the percentage of circulating $PD\text{-}1^+$Tfh cell and the severity of autoimmune diseases. Zhu et al. [28] showed that the percentage of $CD3^+CD4^+$ $CXCR5^+PD\text{-}1^+$ T cells were significantly higher in patients with autoimmune thyroid disease (AITD) than that in HC and were correlated positively with the levels of serum autoantibodies. But, Wang et al. [20] found that the percentages of $CD3^+CD4^+CXCR5^+PD\text{-}1^+$ T cells were correlated negatively with the levels of serum RF and treatment with DMARDs. Our results indicate that % $CD4^+CXCR5^+PD\text{-}1^{hi}$ cell positively correlated with multiple clinic variables, including serum level of CRP, ESR, RF, anti-CCP Ab, IgG and DAS28 index. Thus, the increase of % $CD4^+CXCR5^+PD\text{-}1^{hi}$ cell may reflect the disease severity of RA.

To further reveal the possible mechanism of this phenomenon, we measured the serum level of sPD-1, sPD-L1 and sPD-L2 in the RA patients. The results show that the level of sPD-1 increased synchronously with % $CD4^+CXCR5^+PD\text{-}1^{hi}$ cells, whereas sPD-L1 and sPD-L2 levels decreased. Because of the relative shortage of the ligands, PD-1 cannot fulfill its inhibitory function on the Tfh cell differentiation. From this point, the increased expression of $PD\text{-}1^+$ cell can be explained as a result of the feedback regulation to cover the shortage of PD-1 ligands or function defects. This possibility is worthy of more investigation in the future.

On the other hand, though Tfr cells are usually marked by $CD4^+CXCR5^+Foxp3^+$ [16, 20, 29, 30], it is also found that

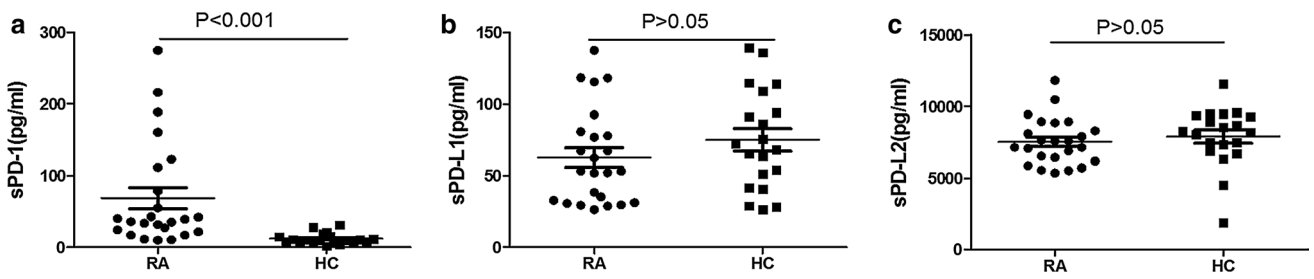

Fig. 6 Levels of sPD-1, sPD-L1 and sPD-L2 in sera of patients with RA and HC

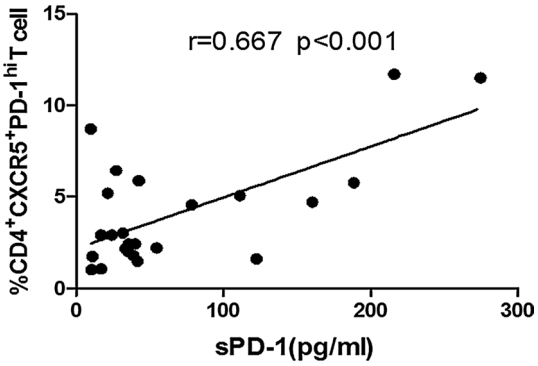

Fig. 7 Correlations between serum sPD-1 levels and %cTfh cells

there is strong negative correlation between the expression of CD127 and Foxp3 on $CD4^+$ T cells in human peripheral blood. The cells purified through $CD4^+CD127^{lo}$ have typical characteristics of Treg cells, especially inhibiting the proliferation of other T cells. Therefore, $CD4^+CXCR5^+CD127^{lo}$ can also be used to represent Tfr cells [31–34].

In our study, we found that $\%CD4^+CXCR5^+CD127^{lo}$ cell increased in the RA patients. Given that Tfh and Tfr cells are reciprocal and antagonistic regulators of GC responses, a balance of their actions is critical for immune homeostasis. A disordered cTfr/cTfh ratio is associated with the development of autoimmune diseases [9]. In consistent with the present result, we found that the ratio of $\%CD4^+CXCR5^+CD127^{lo}/\%CD4^+CXCR5^+PD\text{-}1^{hi}$ cells decreased in the RA patients, indicating an imbalance between cTfh and cTfr cells. The ratio of $\%CD4^+CXCR5^+CD127^{lo}/\%CD4^+CXCR5^+PD\text{-}1^{hi}$ cell correlated inversely with the levels of serum CRP, ESR, RF, anti-CCP Ab, IgG and DAS28 index in RA patients, suggesting that the cTfr/cTfh ratio can be used as a biomarker for the evaluation of disease severity in the RA patients.

There are still some limitations in the present study. The first is the relatively small sample size, which may lead to the no statistical differences when comparing the levels of sPD-L1 and sPD-L2 in different groups. These data may be confirmed in larger-scale studies. Secondly, we did not perform the subset frequencies and the phenotypic characterizations of Tfh cells and Tfr cells in the lymphoid tissues and pathological tissues of RA patients although recent reports have indicated that circulating Tfh cells derived from GC-Tfh cells and shuffling between peripheral blood and lymphoid tissue [35]. Thirdly, functional studies of how Tfh cells and Tfr cells modulate GC reactions and B cells response are still required to uncover the precise molecular mechanisms during the pathogenic process of RA. Finally, though we analyzed the differences between circulating Tfh and Tfr among patients with and without methotrexate treatment, the synergic effect of other treatments cannot be ignored.

Taken together, our study described the subset distribution and phenotypes of cTfh cells and Tfr cells and their relationships with clinical indicators. Our study indicates that unbalanced circulating Tfr/Tfh ratio and aberrant distribution of effector/resting phenotypes of Tfh cells or Tfr cells may contribute to the immunopathogenesis of RA. The Tfr/Tfh ratio may serve as a useful biomarker for RA patients, leading to new strategies for tailoring treatment of RA.

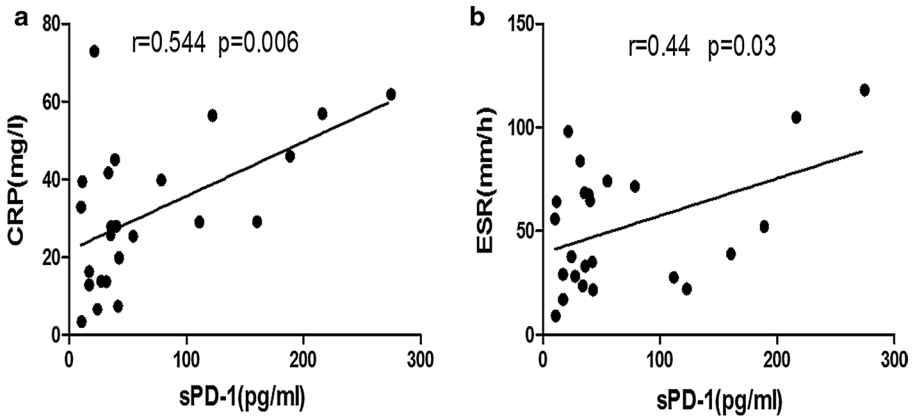

Fig. 8 Correlations between serum sPD-1 levels and clinical parameters. The correlation between serum sPD-1 levels and CRP in RA patients (**a**); the correlation between serum sPD-1 levels and ESR in RA patients (**b**)

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The studies have been approved by the ethics committee of the first affiliated hospital of China Medical University. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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