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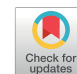
An imbalance between blood CD4$^+$CXCR5$^+$Foxp3$^+$ Tfr cells and CD4$^+$CXCR5$^+$Tfh cells may contribute to the immunopathogenesis of rheumatoid arthritis



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ABSTRACT

Background: Follicular helper T (Tfh) cells are a subgroup of activated CD4$^+$ T cells which can assist the formation and maintenance of germinal centers. Follicular regulatory T (Tfr) cells are a new class of regulatory T cells which play a major role in suppressing cells in humoral immunity. In contrast to the role of Tfh cells, Tfr cells can inhibit the function of Tfh cells and B cells. Imbalance of blood Tfr/Tfh ratio resulted in the expansion of auto-reactive B cells and auto-antibody production (). However, the effect of Tfr cells and Tfh cells in the pathogenesis of RA (rheumatoid arthritis) is unclear. The purpose of this study was to investigate the function of Tfr cells and Tfh cells in the pathogenesis of RA.

Methods: We recruited 20 patients fulfilled the the American College of Rheumatology diagnosis criteria and 20 healthy controls (HCs). The number of CD4$^+$CXCR5$^+$Foxp3$^+$ Tfr cells and CD4$^+$CXCR5$^+$ Tfh cells in 20 RA patients were measured by flow cytometry analysis. Furthermore, the correlations between the Tfr/Tfh ratio and the characteristic clinical parameters were assessed. The serum levels of IL-21(interleukin-21), CXCL13 (chemokine (C-X-C motif) ligand 13) and TGF-β (Transforming growth factor-β) were measured by ELISA. The formation of ectopic germinal center (GC) of synovial membrane was examined by H&E staining. The transcriptional levels of CXCR5 (C-X-C chemokine receptor type 5), CXCL13, ICOS (inducible co-stimulater) and TGF-β mRNA were also analyzed. In addition, the expression of Bcl-6 (B-cell lymphoma 6), CXCR5, CXCL13 and ICOS in synovial membrane were examined by immunohistochemistry.

Results: RA patients had more Tfh cells in peripheral blood, conversely, the frequency of blood Tfr cells ($p < 0.05$) and the ratio of Tfr/Tfh were significantly decreased compared to healthy controls ($p < 0.05$, $p < 0.01$). Furthermore, the ratio of Tfr/Tfh was negatively correlated with values of ESR (r = -0.57, $p < 0.05$), RF (r = -0.5275, $p < 0.001$), CRP (r = -0.4486, $p < 0.001$), IgG (r = -0.4631, $p < 0.05$), DAS28 scores (r = -0.5645, $p < 0.01$), as well as the levels of IL-21(r = -0.7398, $p < 0.01$), CXCL13 (r = -0.4832, $p < 0.05$). However, the ratio of Tfr/Tfh was positively with the serum level of TGF-β (r = 0.5115, $p < 0.05$). Higher mRNA expression of CXCR5, CXCL13, ICOS and lower TGF-β mRNA expression were observed in RA patients. The serum expression level of IL-21, CXCL13 was significantly increased and expression of TGF-β was significantly decreased in RA patients. Furthermore, ectopic germinal center formation and higher expression of Bcl-6, CXCR5, ICOS, CXCL13 in the synovial membrane of the joints in RA patients were observed.

Conclusions: The decreased blood CD4$^+$CXCR5$^+$Foxp3$^+$ Tfr cells/CD4$^+$CXCR5$^+$ Tfh cells may be responsible for the immunopathogenesis of RA.

1. Introduction

Rheumatoid arthritis (RA) is a chronic, inflammatory systemic autoimmune disease characterized by multiple joints of the hand and foot joints, symmetry, invasive joint inflammation, and serum rheumatoid factor, CRP (C-reactive protein), anti-CCP antibody positive. However, the immunopathogenesis of RA is still unclear. Numerous studies have shown that multiple immune cell interactions play an important role in RA (Li et al., 2019), our previous results showed that the imbalance between Treg/Th17 cells was involved in RA pathogenesis (Ma et al.,

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2017). Follicular help T (Tfh) cells are a type of $CD4^+$T cells which mainly expresses transcription factor B-cell lymphoma 6 (Bcl-6), surface molecules such as chemokine (C-X-C) receptor 5 (CXCR5), CD40 ligand, inducible T-cell costimulator (ICOS), programmed cell death protein-1 (PD-1) and cytokines including IL-21, IL-10 and IL-6 (Qiu et al., 2017; Deng et al., 2019). Tfh cells involved in assisting the germinal center B cell activation, proliferation and differentiation into plasma cells (Kurata et al., 2019). Our previous study found that RA patients had a higher percentage of circulating $CD4^+PD\text{-}1^+CXCR5^+$ Tfh cells and higher serum level of IL-21, more importantly, either the frequency of Tfh cells or the expression of IL-21 was associated positively with higher values of rheumatoid factor (RF) and anti-cyclic citrullinated peptide antibodies (ACPA) than those in HCs (Cao et al., 2019). The activated Tfh cells in the peripheral blood may be responsible for the development of rheumatoid arthritis.

Recently, a subset of Tfh cells originate from natural regulatory T cells expressing $Foxp3^+$ was found in germinal centers (GC). Since Foxp3 is expressed in Treg cells, this $CD4^+CXCR5^+Foxp3^+$ subpopulation is called follicular regulatory T (Tfr) cells (Chung et al., 2011). Tfr cells express CXCR5, ICOS, Foxp3, producing TGF-β, IL-10 and other cytokines. It is reported that Tfr cells have a negative regulatory effect on Tfh cells in the follicle, reducing the number of antibodies. Several studies have shown that the imbalance of Tfr/Tfh cells may lead to a large number of self-reactive autoantibodies product, which contributes to the development of autoimmune diseases (Niu et al., 2018; Wang et al., 2019; Zhou et al., 2019a,). The potential functions of follicular regulatory T (Tfr) cells and related cytokines in rheumatoid arthritis (RA) have not been fully elucidated. In the present research, we aimed to investigate whether the imbalance between blood $CD4^+CXCR5^+Foxp3^+$ Tfr cells and $CD4^+CXCR5^+Foxp3^-$ Tfh cells contribute to the pathogenesis of rheumatoid arthritis, as well as their secreting cytokines. Our research indicated decreased frequency of Tfr cells in the peripheral blood of RA patients and the ratio of Tfr/Tfh was negatively correlated with clinical parameters such as DAS28 scores, RF, CRP, ESR and key cytokines IL-21, however, the ratio of Tfr/Tfh was positively with the serum expression of TGF-β.

All studies were approved by the Ethics Committee of Ningxia Medical University.

2. Methods

2.1. Chemicals

To measure the expression of different molecules on Tfh or Tfr cells, cells were stained with monoclonal antibodies to Percp-Cy5.5-CD4 (BD Biosciences,Cat No.560650), AF488-CXCR5 (BD Biosciences,Cat No.558112), PE-Foxp3 (invitrogen Corporation,Cat No.4329354). The monoclonal antibodies Bcl-6 (M00142), CXCR5 (ab203212), CXCL13 (bs-2553R) were purchased from Boster Biological Technology CO.LTD. (Beijing, China), Abcam (Cambridge,UK) and BEIJING BIOSYNTHESIS BIOTECHNOLOGY CO., LTD.ELISA kits for the detection of IL-21 (JL19266−96T), TGF-β (ml064258) and CXCL13 (70-EK1105−96) were bought from Shanghai Jianglai industrial Limited By Share Ltd,Shanghai Enzyme-linked Biotechnology Co.,Ltd.(Shanghai,China) and MULTISCIENCES (LIANKE) BIOTECH,CO.,LTD. Lymphocyte Separation Medium (Human) were bought from Beijing Solarbio Science & Technology Co., Ltd. RNApure Tissue&Cell Kit (CW0584S) was from CoWin Biosciences (Beijing, China). PrimeScript™ RT reagent Kit (Perfect Real Time RR036A) and TB Green® Premix Ex Taq™ II (Tli RNaseH Plus RR820A) was bought from Takara Biomedical Technology Co., Ltd.

2.2. Patients

In this study, patients with first onset or who had not used anti-rheumatic drugs within 3 months before admission were taken as the research object.

Table 1
Characteristics of RA patients and healthy controls.

	RA	HC
Age (years)	52.2 ± 14.24	61.55 ± 11.42
Gender (female/male)	16/4	14/6
ESR (mm/h)	49.55 ± 26.02	4.55 ± 2.99
CRP (mg/L)	31.58 ± 31.50	0.94 ± 0.78
DAS28	8.65 ± 3.23	–
RF(IU/mL)	124.63 ± 52.77	6.63 ± 2.00
Anti-CCP(RU/mL)	96.69 ± 83.79	–
WBC (10^9/L)	6.68 ± 3.09	6.43 ± 1.17
NEUT (%)	62.78 ± 14.86	54.53 ± 6.94
LYM (%)	24.80 ± 9.93	37.30 ± 7.06
Morning stiffness is more than or equal to 1h	11/20	–
Hep2-ANA	8/20	–
ENA-AbRo	6/20	–
IgA(g/L)	3.40 ± 1.10	2.50 ± 1.06
IgG(g/L)	14.68 ± 3.25	12.82 ± 2.18
IgM(g/L)	1.33 ± 0.45	0.97 ± 0.55

20 patients with rheumatoid arthritis and 20 healthy controls (HCs) examined in the General Hospital of Ningxia Medical University from December 2018 to May 2019 were enrolled in this study (Aletaha et al., 2010). The inclusion or exclusion criteria of RA and HCs were reported in our previous study (Cao et al., 2019). The peripheral venous blood remaining after conventional laboratory tests was used in this study, which was admitted in line with the ethical standards of the Declaration of Helsinki. Clinical indicators of patients and HCs are shown in Table 1.

2.3. Flow cytometric analysis

3 mL of peripheral blood was drawn from patients and healthy controls and placed in a test tube containing EDTA. Peripheral blood mononuclear cells (PBMCs) were isolated by Lymphocyte Separation Medium (Human P8610, Solarbio, China). PBMCs at 2×10^6/tube were stained with CD4-Percp-Cy5.5 (Cat No.560650), CXCR5-AF488 (Cat No.558112) (BD Bioscience,USA) for 30 min. Surface-stained cells were permeabilized and fixed (invirogen 00,552,300) then stained with Foxp3-PE (invitrogen, Cat No.4329354). After washing with phosphate-buffered saline (PBS), the cells were analyzed on a BD fluorescence activated cell sorter (FACS) Accuri C6.

2.4. ELISA analysis of serum cytokines

The serum levels of serum IL-21, TGF-β and CXCL13 in RA patients and healthy controls were detected by ELISA kit according to the manufacturer's instructions (Shanghai Jianglai industrial Limited By Share Ltd. Shanghai, China).The absorbance was measured spectrophotometrically at 450 nm using a microplate reader and plotted against a standard curve with standard levels expressed as pg/mL.

2.5. RNA extraction and quantitative real-time PCR (RT-qPCR)

Total RNA was extracted from peripheral blood mononuclear cells in patients and HCs by RNA pure Tissue&Cell Kit (CW0584S). For RT-PCR, cDNA was synthesized from total RNA using PrimeScript™ RT reagent Kit (Takara Biomedical Technology RR036A). Sequences of primers for PCR assay are listed in Table 2. Quantitative real-time PCR (qPCR) were performed in triplicates using TB Green® Premix Ex Taq™ II (Tli RNaseH Plus RR820A) as following: 95 °C for 30 s, 40 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 34 s. Relative mRNA expression in peripheral blood mononuclear cells was calculated using the comparative $2^{-\Delta\Delta Ct}$ method.

Table 2
Sequences of primers for RT-qPCR.

Primer name	Sequences (5′ to 3′)	Number of bases
Human-TGF-β-F	CTGTACATTGACTTCCGCAAG	21
Human-TGF-β-R	TGTCCAGGCTCCAAATGTAG	20
Human-ICOS -F	CTCTGCGATCTCACTAAGACAA	22
Human-ICOS -R	GGTTGCAGAAGTAATAGTTGGC	22
Human-CXCL13 -F	CTTGGTAGAGAAGCTTGAGACA	22
Human-CXCL13 -R	GGTGTTTTGATCTTTAGAGGCC	22
Human-CXCR5 -F	ATCGCCGTGGACCGCTACC	19
Human-CXCR5 -R	CTGGCAAGGCAAGGAGGAAGC	21
HS-ACTB-F	CCTGGCACCCAGCACAAT	18
HS-ACTB-R	GGGCCGGACTCGTCATAC	18

2.6. Hematoxylin and eosin staining

The synovial tissues of patients and HCs were dissected and immediately placed in 4% paraformaldehyde for 24 h, embedded in paraffin, stained with hematoxylin and eosin (HE), and photographs were captured using an OLYMPUS microscope. Assess the histopathological changes and observe the formation of ectopic germinal centers.The synovial tissue sections were observed and evaluated blind.

2.7. Immunohistochemistry

Tissue sections of three patients and three normal people (each having a thickness of 4 μm) were dewaxed in xylene and then immersed in alcohol and antigen repaired with high pressure for 15 min followed by incubation in H_2O_2 for 10 min to inactivate endogenous peroxidase. Sections were then incubated with antibodies against ICOS (1:100), CXCR5 (1:100), CXCL13 (1:200) and Bcl-6 (1:300) respectively at 4 °C overnight. Immunostaining was performed by using the Universal two-step test kit according to the manufacturer's instructions. Each stained section was evaluated by two independent researchers. Positive staining as a percentage of total area was determined by ImageJ.

2.8. Statistical analysis

Quantitative variables were presented are analyzed as mean ± standard deviation. All data and correlation were analyzed with GraphPad Prism version 6.0. The comparison between the two groups was calculated by t test, and the correlation analysis was performed by Pearson's correlation coefficient analysis.A two-sided p value < 0.05 were statistically considered to be significantly different.

3. Results

Clinical characteristics of study subjects

This study used 20 RA patients and 20 healthy controls. The clinical characteristics are listed in Table 1. No significant difference in age and gender distribution between patients and healthy controls.

3.1. The percentage of blood $CD4^+CXCR5^+$ Tfh cells were increased and $CD4^+CXCR5^+Foxp3^+$ Tfr cells were decreased in RA patients

To study the potential role of Tfr cells in the pathogenesis of RA, 20 RA patients and 20 healthy controls with matched age and gender were recruited. The percentage of peripheral blood $CD4^+CXCR5^+Foxp3^+$ Tfr cells and $CD4^+CXCR5^+$ Tfh cells in healthy controls and RA patients was detected by flow cytometry analysis, 50,000 events for each sample were acquired in the CD4 lymphocyte gate (Fig.1A). We found that the percentage of Tfr cells was significantly decreased in RA patients, while the percentage of Tfh cells was significantly increased which compared to healthy controls (Fig.1B), suggesting that the Tfr cell subset is indeed under-represented in these patients and involved in the pathogenesis of RA.

3.2. Negative correlation between Tfr/Tfh ratio and clinical parameters in RA patients

Our previous study found that the percentage of circulating $CD4^+PD\text{-}1^+CXCR5^+$ Tfh cells was positively correlated with the values of ESR, RF, CRP and ACPA. To investigate whether Tfr cells correlated with autoantibodies, inflammatory markers and disease activity,we conducted correlation figures. We found that the Tfr/Tfh ratio was negatively correlated with the values of DAS28 scores (r = -0.5645, $p < 0.01$), IgG (r = -0.4631, $p < 0.05$), RF (r = -0.5275, $p < 0.05$) (Fig.2D,2E,2B) and inflammation markers ESR (r = -0.57, $p < 0.05$), CRP (r = -0.4486, $p < 0.05$) in RA patients (Fig.2C,2A). In addition, we also found that CD4 $^+$ CXCR5 $^+$ Foxp3 $^+$ Tfr cells were negatively correlated with DAS28 scores (r = -0.5334, $p < 0.05$), IgG (r = -0.4870, $p < 0.05$), RF (r = -0.4612, $p < 0.05$) (Fig.2I,2J,2G) and inflammation markers ESR (r = -0.6008, $p < 0.05$), CRP (r = -0.4640, $p < 0.05$) in RA patients (Fig.2H,2F).These findings indicated that Tfr cells and Tfr/Tfh ratio were dysregulated in RA patients, Tfr cells might be involved in production of pathogenic auto-antibodies and inflammation.

3.3. Decreased Tfr/Tfh ratio are negatively correlated with higher levels of IL-21, CXCL13 and positively with lower level of TGF-β in RA patients

To know whether the IL-21, CXCL13 and TGF-β play important roles in the pathogenesis of RA, we examined their serum expression level in the RA patients and health controls by ELISA. The results showed that the serum levels of IL-21 and CXCL13 in RA patients were significantly higher than those of healthy controls ($p < 0.01$) (Fig. 3A,3C). On the contrary, the serum level of TGF-β was significantly lower than that in HCs ($p < 0.01$) (Fig.3B). We next studied whether the deregulated Tfr/Tfh ratio wasassociated with the signature cytokines, we found that the blood Tfr/Tfh ratio inversely related to the concentration of IL-21 (r = -0.7398, $p < 0.01$) or CXCL13 in the serum (r = -0.4832, $p < 0.05$), while the Tfr/Tfh ratio was positive association with the concentration of TGF-β (r = 0.5115, $p < 0.05$) (Fig.3D, 3F,3E).

3.4. Decreased mRNA expression of TGF-β and elevated mRNA expression of ICOS, CXCR5 and CXCL13 in RA Patients

Tfr cells are a specialized population of effector T regulatory cells which suppresses B-cell responses in the germinal center (GC). Tfr cells express typical markers such as Foxp3, ICOS, and CXCR5 that directs them to the GC. To further examine whether the regulation of Tfr cells to Tfh cells in RA patients are important for RA pathogenesis, the mRNA expression of TGF-β, ICOS, CXCR5 and CXCL13 in RA patients and healthy controls were measured by RT-qPCR. Our results showed that TGF-β mRNA expression in RA patients was lower than that in healthy controls (Fig. 4D), while the mRNA expression of ICOS, CXCR5 and CXCL13 were elevated in RA patients (Fig. 4A,B,C).

3.5. Formation of ectopic germinal center in the synovium of joint lead to invasive joint inflammation in RA patients

In the GC, B cells interact closely with Tfh cells, which facilitate B-cell activation, somatic hypermutation, affinity maturation, class switch recombination, and differentiation of B cells into memory B cells and plasma cells. As GC reactions take place in lymphoid tissues and ectopic (or tertiary) lymphoid structures, we further studied synovial tissue of joint biopsies from RA patients and healthy controls. Synovial tissue hyperplasia was observed by HE staining in the synovial membrane of RA group, in which we found that a large number of inflammatory cells infiltrated (Fig. 5A,5B), and ectopic germinal center formation (Fig.5C,5D). Meanwhile, we used immunohistochemistry to detect the expression of CXCR5, CXCL13, ICOS, and Bcl-6 in the synovium of the

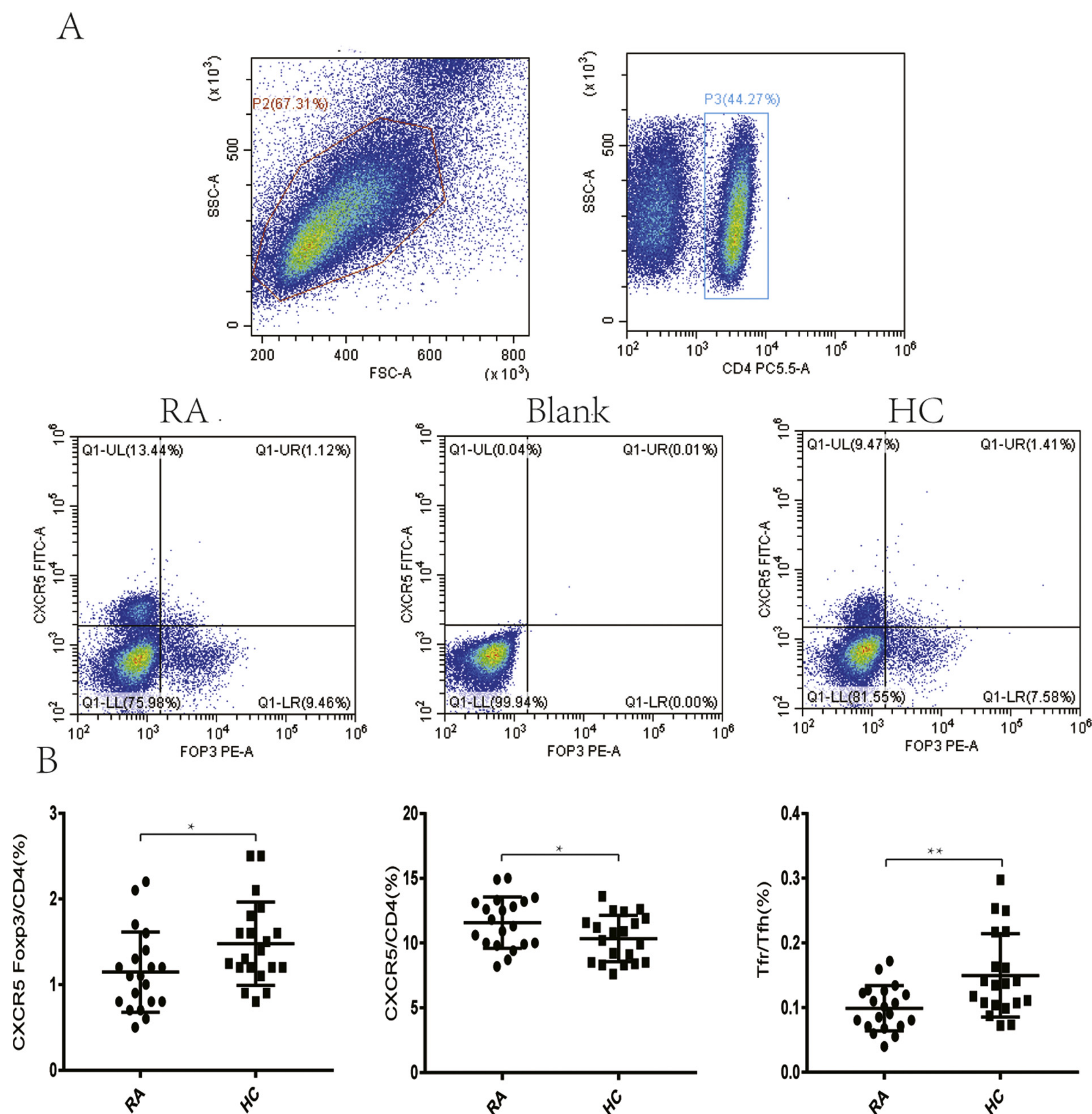

Fig. 1. The frequency of Tfh cells and Tfr cells in RA patients was observed by Flow cytometry analysis. PBMCs from RA patients and HCs were stained with Percp-Cy5.
5-CD4, AF488-CXCR5, PE-Foxp3. A.The proportion of $CXCR5^+Foxp3^+$ cells in $CD4^+$ T cells in PBMCs of RA patients and healthy controls (Top right is the percentage of Tfr (CD4 + CXCR5 + Foxp3 +) cells. Upper left is the percentage of Tfh (CD4 + CXCR5 + Foxp3-) cells); B.Horizontal bars indicate the mean and error bars represent the SEM. Frequency of Tfh cells (*$p < 0.05$), Tfr cells (*$p < 0.05$), Tfr/Tfh (**$p < 0.01$) cells in RA patients and healthy controls.

joint (Fig. 6A-D). The results showed that the expression of CXCR5, CXCL13, ICOS and Bcl-6 in the synovium of RA group significantly increased compared with the normal group. It is reported that peripheral blood Tfr/Tfh ratio caused pathological lymphocytic infiltration in RA target organ. As the Tfr/Tfh ratio may predict the outcome of GC reactions,we hypothesized that patients with autoimmune diseases with abnormal GC reactions leading to autoantibody production may have an altered Tfr/Tfh ratio.

4. Disscusion

To further verify whether Tfh cells and Tfr cells are involved in the pathogenesis of RA,our previous studies have determined that Tfh cells and cytokine IL-21 are thought to be crucial in the induction and progression of RA, which may contribute to the pathogenesis of RA (Cao et al., 2019). However, how Tfh cells and IL-21 promote the development of RA remains largely unclear. In this study, we found that peripheral blood $CD4^+CXCR5^+Foxp3^-$Tfh cells were increased,however $CD4^+CXCR5^+Foxp3^+$Tfr cells and Tfr/Tfh ratio were decreased. In addition, we found $CD4^+CXCR5^+Foxp3^+$Tfr cells and the ratio of Tfr to Tfh were negatively correlated with clinically relevant immune markers. This observation is consistent with the study by Niu et al (2018). However, our findings are different from they found by Wang et al (2019). Other research showed that the percentage of Tfr ($CD4^+CXCR5^+$ $CD127^{lo}$) in $CD4^+$ T cells were significantly higher than that in HC,it may be related to our definition of Tfr.Because Tfr cells have the double characteristics of Tfh cells and regulatory T cells, so we selected Foxp3, a characteristic transcription factor for regulatory

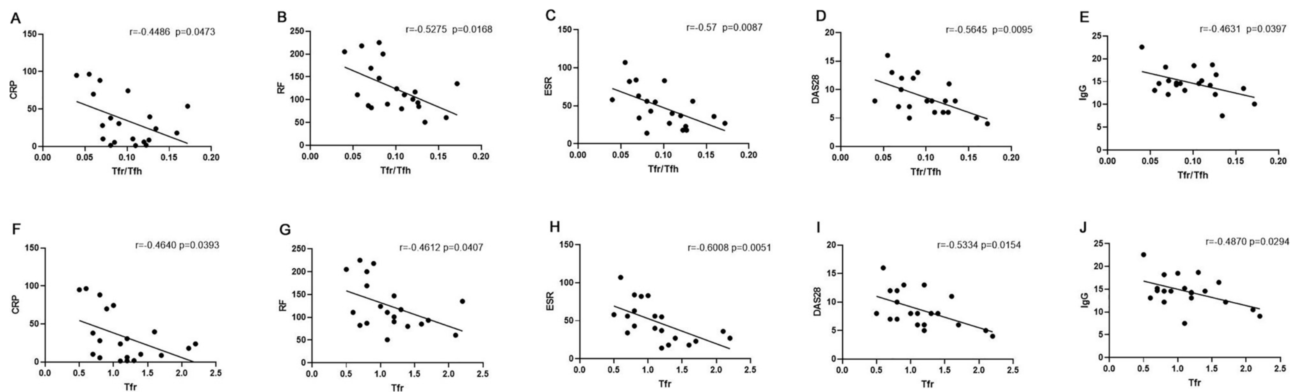

Fig. 2. Correlation analysis of clinic pathological features of RA patients with the ratio of Tfr/Tfh and Tfr cells. (A)The ratio of Tfr/Tfh was negatively correlated with the value of CRP. (B-E) Correlation between the ratio of Tfr/Tfh and RF, ESR, DAS28and IgG(F)The Tfr cells was negatively correlated with the value of CRP.(G-J)Correlation between the Tfr cells and RF, ESR, DAS28 and IgG.The ratio of Tfr/Tfh and Tfr cells were negatively correlated with CRP, RF, ESR, DAS28 and IgG. *$p < 0.05$, **$p < 0.01$ compared with healthy controls.

T cells, and the special surface marker CXCR5 of Tfh cells.

Although many literatures define CD4 + CXCR5 + ICOS +,CD4 + CXCR5 + PD-1 + as Tfh cells,we can also say that CD4 + CXCR5 + T cells express ICOS, PD-1, which also expresses the cytokine IL-21 and the transcription factor Bcl-6,indicating that we can define CD4 + CXCR5 + T cells as Tfh cells.In addition,the ratio of Tfr to Tfh cells is positively correlated with RF and IgG in RA patients, which suggests that the imbalance of the ratio of Tfr to Tfh cells may be involved in the production of autoantibodies. Moreover, the ratio of Tfr to Tfh cells is related to DAS28, CRP, and ESR, indicating that either the Tfh cells or Tfr cells could aggravate arthritis.

CXCR5,Bcl-6 and ICOS are essential for the development of Tfh cells, which is critical for the production and survival of Tfh cells, the formation of germinal centers, and the production of specific antibodies (Pedros et al., 2018). Moschovakis et al. (Moschovakis et al., 2017) showed that CXCR5 was an absolutely necessary factor to induce inflammatory autoimmune arthritis,CXCR5 deficient mice and mice selectively lacking CXCR5 on T cells were completely resistant to CIA.It has also been reported that the mice with CXCR5 deficient Treg cells have more GC (Wollenberg et al., 2011).This suggests that Tfh and Tfr cells play a crucial role in maintaining immune tolerance and preventing autoimmune response

Jogdand et al. (Jogdand et al., 2016a) observed that over-expression of Bcl6 and ICOS can upregulate the expression of CXCR5 on the surface of Tfh cells, promote the differentiation of Tfh cells. The observation from Chu et al. (Chu et al., 2014) showed that Tfh cells and their surface molecules such as CD4, ICOS and CXCR5 were significantly increased in synovial tissue of RA patients. The result is consistent with our findings. In the present study, we found the expression of ICOS and Bcl-6 of synovial membrane in RA patients was significantly higher than that in the control group. This suggests that the formation of ectopic germinal center and infiltration of Tfh cells may be involved in the development of RA. Although many researchers have confirmed the role of Bcl-6 and ICOS in autoimmune diseases and infectious diseases, it is still unknown

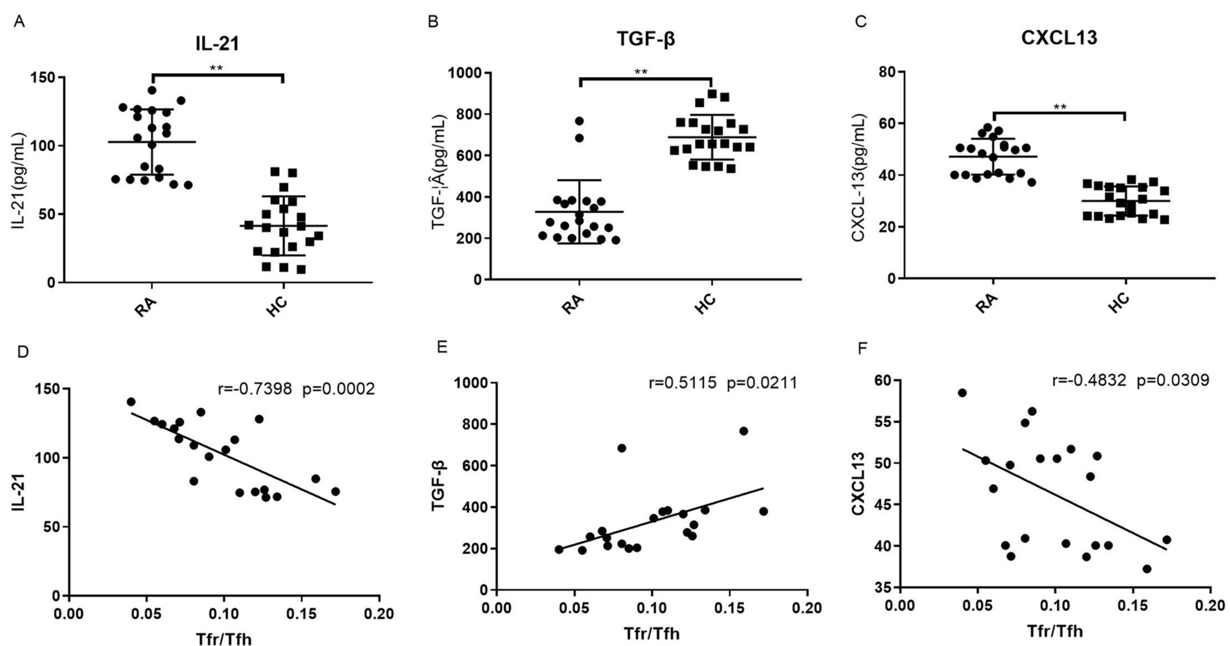

Fig. 3. The levels of Tfh and Tfr cell-associated cytokines IL-21, TGF-β and CXCL13 in serum of RA patients and healthy controls were measured by ELISA. (A) Serum level of IL-21 (**$p < 0.01$). (B)Serum level of TGF-β (**$p < 0.01$). (C)Serum level of CXCL13 (**$p < 0.01$). (D)The ratio of Tfr/Tfh was correlated negatively with the level of serum IL-21 in RA (r = -0.7398, $p < 0.001$). (E)The ratio of Tfr/Tfh was correlated positively with the level of serum TGF-β in RA (r = 0.5115, $p < 0.05$). (F)The ratio of Tfr/Tfh was correlated negatively with the level of serum CXCL13 in RA patients (r = -0.4832, $p < 0.05$). *$p < 0.05$, **$p < 0.01$ compared with healthy controls.

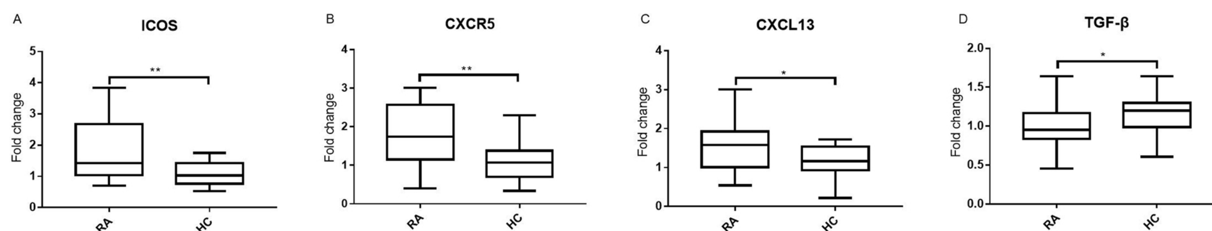

Fig. 4. RT-qPCR was used to detect the mRNA expression of Tfh and Tfr cells-associated transcription factors CXCR5, TGF-β, ICOS and CXCL-13 in peripheral blood PBMCs of RA patients and healthy controls. (A)ICOS mRNA expression by RT q-PCR (**$p < 0.01$). (B)CXCR5 mRNA expression by RT-qPCR (**$p < 0.01$). (C)CXCL13 mRNA expression by RT-qPCR (*$p < 0.05$). (D)TGF-β mRNA expression by RT-qPCR (*$p < 0.05$). *$p < 0.05$, **$p < 0.01$ compared with healthy controls.

the detailed immunological mechanism in RA. This study provides a basis for further exploration of the pathogenesis of RA and treatment of RA.

To further reveal the possible mechanism of Tfr and Tfh cells in RA patients, we measured serum levels of TGF-β and IL-21. IL-21 and TGF-β have also been used to symbolize the number of Tfh and Tfr cells.Studies have shown that abnormal expression of IL-21 is associated with a variety of autoimmune diseases (Zhou et al., 2019a,).TGF-β is one of the key factors for Tfr cells to suppress immune suppression, which is mainly secreted by Treg cells. It is reported that TGF - β secreted by Tfr cells can inhibit the function of Tfh cells (Li et al., 2016). We found that IL-21 was significantly increased and Tfr cells were significantly reduced in RA patients. Furthermore, the ratio of Tfr to Tfh cells was negatively correlated with IL-21 and positively correlated with TGF-β, which indicated that TGF-β could inhibit Tfh cell-mediated germinal center immune response and antibodies production through repressed the secretion of IL-21. In a way, it also balances Tfr cells and Tfh cells. At the same time, increased serum levels of IL-21 and decreased levels of TGF-β may be related to the pathogenesis of RA.

Recently, more and more research have focused on the relationship between Tfr and Tfh in autoimmune diseases. Xu et al. (2019) showed that Tfr cells were significantly decreased in type 1 diabetes (T1D). Xu et al. (2017) demonstrated decreased frequency of Tfr cells and increased Tfh/Tfr ratio in SLE patients.These results are consistent with our results in RA. Therefore, the dysregulation of Tfr cells and Tfh cells may contribute to the pathogenesis of autoimmune diseases (Fig. 7).

Our research has a lot of limitations. The number of patients in our study is relatively small and more in-depth research should be conducted to better support our conclusions. Due to the small number of Tfh and Tfr cells, it is difficult to culture in vitro. Therefore, PBMC was selected for related experiments in this experiment.our team needs to conduct further research at the level of Tfh and Tfr in the future.And the

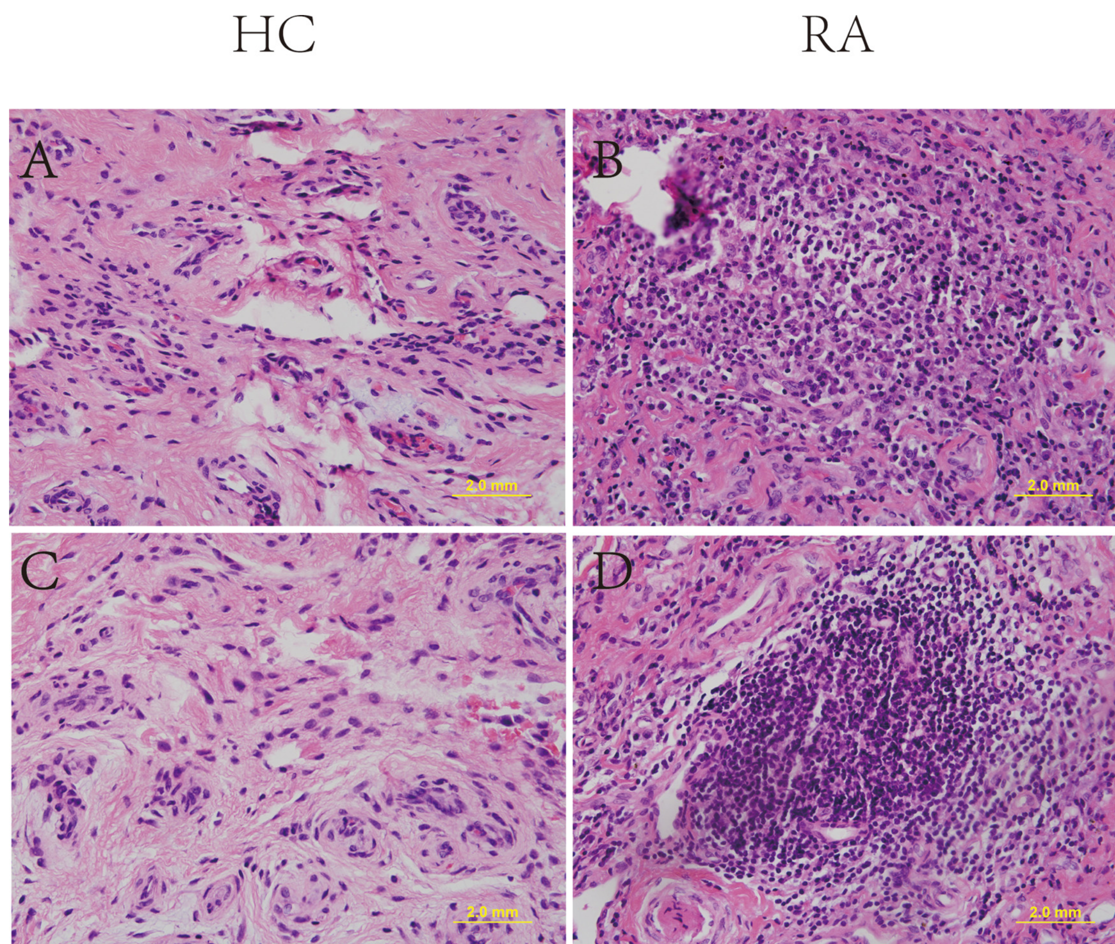

Fig. 5. HE staining of synovial tissue in RA patients and healthy control, we found that the synovial membrane of RA patients has a large number of inflammatory cell (B) infiltration with ectopic germinal center formation(D), compared with healthy controls (A, C).

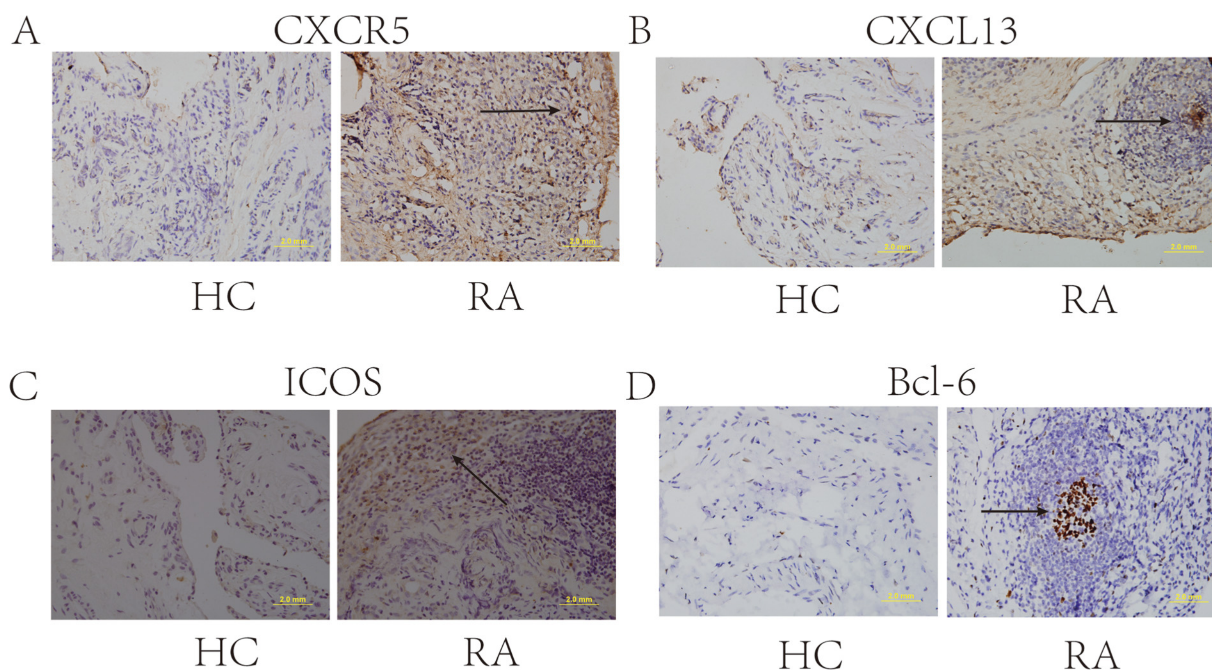

Fig. 6. Immunohistochemical analysis of synovial tissue in RA patients and HCs. (A) Immunohistochemistry was used to detect the expression of CXCR5 in synovial tissue of RA patients. (B) Positive CXCL13 immunostaining in rheumatoid synovium tissue.(C)Immunohistochemistry of ICOS in synovium tissue from RA Patient.(D) Immunohistochemistry was used to detect the expression of Bcl-6 in synovial tissue of RA patients.(×400 magnification, Scale Bar = 2.0 mm).

chemokine receptors CXCR3 and CCR6 are commonly used markers to define Tfh subsets.That is Tfh1, Tfh2 and Tfh17. These Tfh subsets have different complementary effects on Tfr cells, which will appear in our future research.

5. Conclusion

Our research data suggested that the Tfr cells and Tfh cells were involved in the pathological progression of RA, the Tfr cells might be responsible for regulating the crosstalk between Tfr cells and Tfh cells in the germinal central and the production of autoantibodies in RA.

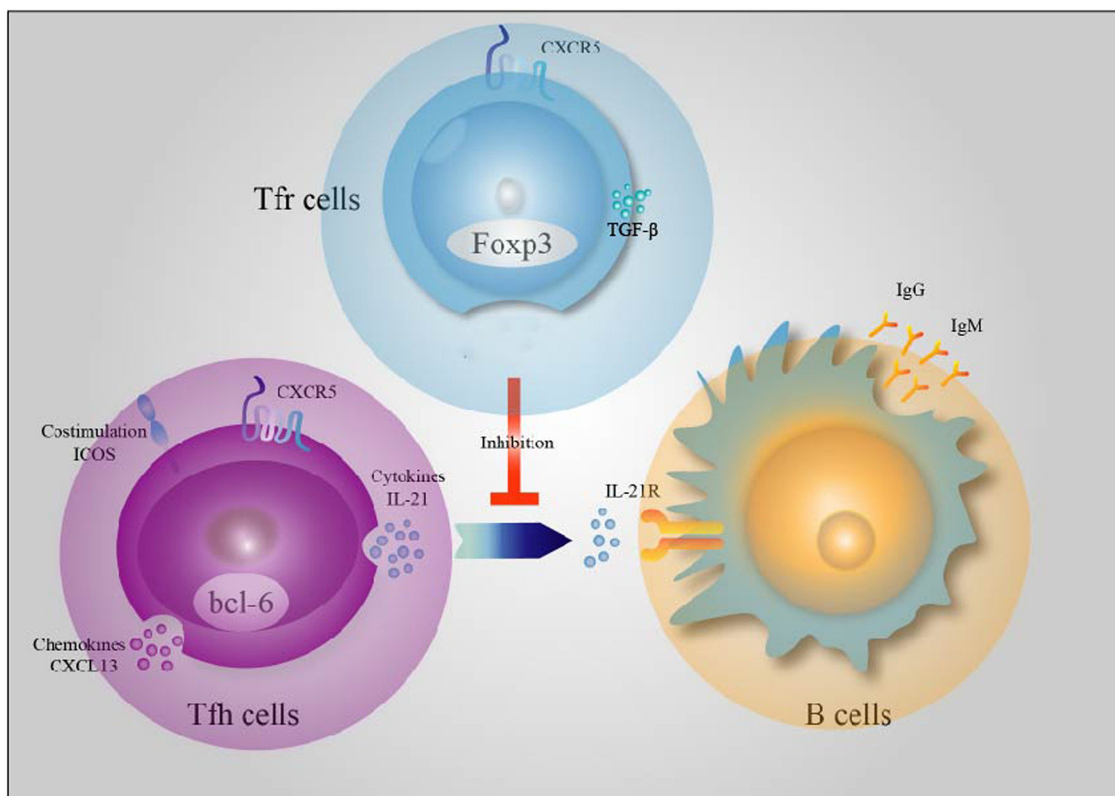

Fig. 7. The regulate mechanism of Tfh cells and Tfr cells in RA patients.

However, the repression mechanisms of Tfr cells, and how Tfr cells regulates Tfh cells and B cells activation and differentiation in GC, needs to be further studied in the future.

Author contributions statement

Yanli Zhang participated in the design of this study. Gan Cao, Peipei Wang, Zhenhua Cui and Xiaoqi Yue contributed to the analysis of the experimental results and the writing of the article. Shuhong Chi and Ailing Ma participated in the collection of specimens.All authors have revised and approved the manuscript.

Ethics statements

This retrospective study was approved by the Ethics Committee of Ningxia Medical University and the methods were carried out in accordance with the approved guidelines. All the patients have been informed and signed informed consent before the experiments.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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