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Mechanism of PD-1/PD-L1 in Regulating cTfr/cTfh Balance in Patients with Rheumatoid Arthritis

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ABSTRACT

Rheumatoid arthritis (RA) is frequent, an imbalance between helper cells (Th) and regulatory T cells (Treg) is the fundamental immunological cause of RA. This study investigates how recombinant human programmed cell death 1 (PD-L1) protein affects circulating T follicular helper (cTfh), circulating T follicular regulatory (cTfr), and their equilibrium.

Magnetic bead sorting was used to select CD4+CXCR5+T cells from RA patients' and healthy individuals' peripheral blood mononuclear cells for in vitro growth. Recombinant human PD-L1 protein stimulated CD4+CXCR5+T cells. Cell counting kit 8 (CCK-8), flow cytometry surface labeling, ELISA, and RT-PCR were used to measure CD4+CXCR5+T cell proliferation inhibition, cTfh and cTfr frequencies, IL-21 expression, and PI3K, AKT, Bcl-6, and Blimp-1 mRNA levels.

The recombinant human PD-L1 protein dose-dependently inhibited the proliferation of CD4+CXCR5+T cells in active RA peripheral blood. However, it has a weaker inhibitory effect on healthy peripheral blood CD4+CXCR5+T cells. PD-L1 protein decreased cTfh in active RA peripheral blood CD4+CXCR5+T overall cultured cells but did not affect cTfr; The cTfr/cTfh ratio increased but did not affect the frequency of cTfh and cTfr in healthy persons' cultured CD4+CXCR5+T cells. PD-L1 protein reduced IL-21 in CD4+CXCR5+T cell culture supernatant from active RA peripheral blood. Recombinant human PD-L1 protein lowered PI3K, AKT, and Bcl-6 mRNA in active RA peripheral blood CD4+CXCR5+T cell culture, including significant differences. But Blinmp-1 mRNA variations were neither substantial nor statistically different.

PD-1/PD-L1 limits cTfh proliferation, differentiation, and activation via the PI3K/AKT signaling pathway regulates its immunological balance with cTfr, and corrects the cTfr/cTfh imbalance by controlling their interaction.

Keywords: Follicular helper T cells; Follicular regulatory T cells; Programmed cell death 1; Rheumatoid arthritis

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INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune condition characterized by persistent, advancing,

erosive, and symmetric polyarthritis.[1] Like other inflammatory disorders, it has a high prevalence worldwide.[2,3] The global prevalence of RA is 0.5-1%. Within 2 years of the onset of RA, approximately 75% of patients experience irreversible joint damage, ultimately leading to joint deformities and even disability, often accompanied by dysfunction of multiple organs and systems throughout the body.[4,5] Currently, there is a widely held notion that the development of rheumatoid arthritis encompasses the involvement of numerous immune functional cells., including B cells, T cells, dendritic cells, and macrophages.[4,6,7] Autoreactive T cells are recognized to play a pivotal role in the development of rheumatoid arthritis since they stimulate macrophages and B cells leading to the production of autoantibodies and pro-inflammatory markers, thereby mediating adaptive immunity. Existing research has demonstrated that the immunological etiology of RA mostly arises from an imbalance in the ratio between regulatory T cells (Treg) and T helper cells (Th). These cells, including Th1, Th17, and T follicular helper cells (Tfh) cells, are well documented in the acceleration of RA progression. Recent studies demonstrated an elevation in the levels of Tfh within the peripheral blood of individuals diagnosed with RA. There is a notable increase in the expression of programmed cell death factor 1 (PD-1) in T follicular helper cells. PD-1 is a member of the CD28 family, weighing approximately 55kDa. PD-1 is shown to be present in T cells, B cells, and monocytes upon activation.[8,9] PD-1 exerts a negative regulatory effect on the signaling of the T cell receptor (TCR) by facilitating the recruitment of Src homologous tyrosine phosphatase 2 (SHP-2), which possesses two domains, to phosphorylated tyrosine residues located in the cytoplasmic area. Programmed death-ligand 1 (PD-L1) and PD-L2 are two ligands that interact with PD-1. PD-L1 is classified as a transmembrane protein of type 1, which inhibits the proliferation and activation of T cells through its interaction with PD-1. Upon forming a complex with PD-L2/PD-L1, PD-1 inhibits the activation of antigen-presenting cells (APCs), B cells, and T cells, hence contributing to the preservation of immunological tolerance. The perturbation of autoimmune tolerance and persistent stimulation of the immune response are important factors in the pathophysiology of rheumatic immunological diseases. Earlier research has indicated that PD-1 plays a significant role in immune-mediated rheumatic diseases, and PD-L1/PD-1 mediated immune abnormalities play a role in the occurrence and the development of RA, Psoriatic arthritis (PsA), Ankylosing spondylitis (AS), systemic lupus erythematosus (SLE), and other diseases. In individuals with rheumatoid arthritis, there is a heightened expression of PD-1 on T cells within the proliferative synovial tissue, joint synovial fluid, and peripheral blood,[8-10] however, PD-L1 is expressed in synovial tissue and synovial fluid,[4,10] suggesting that PD-L1/PD-1 can play a role in the pathogenesis of RA. Further research has shown that researchers have simulated the PD-L1/PD-1 pathway by injecting PI3K inhibitors in collagen-induced arthritis (CIA) mice that have knocked out the PD-1 gene to weaken the PI3K/AKT signaling pathway; Studies report that activated PD-1 by intraperitoneal injection of recombinant PD-L1 protein into CIA mice, and the results indicated a marked decrease in mouse arthritis activity, indicating that the interplay between PD-L1 and PD-1 delayed the progression of CIA. Furthermore, following the inhibition of signaling pathway of the PD-L1/PD-1 in T cells during in vitro tests, there was a noted rise in teh cels activation, as well as an apparent imbalance in the Treg cels activation. According to recent research, it has been proposed that the PD-1 negative regulatory signaling pathway inside the inflammatory milieu of RA could potentially be inhibited by sPD-1.[11] Mouse experiments have shown that the attachment of the sPD-1 molecule to PD-L1 blocks the original negative regulatory signaling pathway, accelerating the pathological progression of CIA mice. Reighard et al. Intended PD-L1 chimeric antigen receptor NK cells, which can selectively eliminate Tfh cells in human mice with lupus-like disease.[12-14]

According to the literature, CD4+CXCR5+ T cells, commonly referred to as Tfh cells, have been identified as playing a significant role in the development of rheumatoid arthritis.[15] Earlier investigations have demonstrated an increase in the concentrations of CD4+CXCR5+ Tfh cells in the peripheral blood of individuals diagnosed with rheumatoid arthritis.[16] Tfh cells exhibit a high expression of PD-1 and actively contribute to B cell maturation, antibody class switching, and the formation of long-lived plasma cells and memory B cells within germinal centers.[10] As such, CD4+CXCR5+ Tfh cells are pivotal in mediating adaptive immunity and the autoimmune response in RA.[15]

The main aim of this study was to investigate the existence and features of CD4+CXCR5+ T cells in the peripheral blood of individuals diagnosed with rheumatoid arthritis. In this study, the researchers conducted in vitro stimulation of recombinant human PD-L1 protein to investigate the alterations in cTfr/cTfh, as well as the participation of two primary transcription factors and potential signaling pathways.

MATERIALS AND METHODS

Study Population and Grouping

The Rheumatology and Immunology Department of the First Affiliated Hospital of China Medical University had 22 hospitalized RA patients from October 2018 to March 2019. ACR 2010 categorization criteria were met by all patients.[18] Exclusion criteria: Female patients with clear evidence of infection within the past month, recent surgery or trauma, pregnancy or breastfeeding, autoimmune diseases in themselves or their immediate family, long-term hormone or immunosuppressant use, and other illnesses. 22 healthy individuals matched for both age and gender to serve as controls in the study. The CD4+CXCR5+T cells in the peripheral blood of the study participant were separated into three groups: control, low-dose (recombinant human PD-L1 protein 2 ug/mL), and high-dose. This study received approval from the Medical Ethics Committee of the First Affiliated Hospital of Jinzhou Medical University**,** and all RA and healthy volunteers signed informed consent forms.

Data Collection

Clinical data of patients based on an electronic medical record system, including patient name, gender, age, ethnicity, duration of morning stiffness, number of patient global assessment of disease activity (PGA), number of tender joints (TJC), swollen joints (SJC), The doctor's overall evaluation of disease activity (EGA) and other clinical manifestations, as well as medication usage were collected. Laboratory data of patients, including hematuria routine, anti-citrullinated peptide antibody (Anti CCP), rheumatoid factor (RF), C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), etc. were also collected. To meet the unified evaluation criteria, fixed doctors completed the collection of clinical data. Based on the above clinical data, the disease activity score simplified disease activity index (SDAI), clinical disease activity index (CDAI), and [the disease activity score in 28 joints, ESR-based or CRP-based, DAS28 (ESR) or DAS28 (CRP)] for 28 joints were calculated.

Samples and Testing

In the morning, 10 mL peripheral whole blood samples were gathered from both rheumatoid arthritis (RA) patients and healthy controls. Peripheral blood mononuclear cells (PBMCs) were isolated using a rinsing solution, and then CD4+T cells were selected using magnetic bead negative sorting, and CD4+CXCR5+T cells were selected through magnetic bead positive sorting. CCK-8 was applied to determine the toxicity of recombinant human PD-L1 protein (Abcam, Cambridge, UK) on CD4+CXCR5+T cells, flow cytometry was performed to calculate the ratio of cTfh and cTfr in peripheral blood after CD4+CXCR5+T cell culture, enzyme-linked immunosorbent assay (ELISA) was performed to measure the concentration of IL-21 in the supernatant of CD4+CXCR5+T cell culture, and RT-PCR was used to detect the levels of PI3K, AKT, Bcl-6, and Blimp-1 in CD4+CXCR5+T cell culture.

Magnetic Bead Sorting of CD4+CXCR5+ T Cells

Peripheral blood mononuclear cells (PBMCs) were separated from whole blood samples by employing a rinsing solution. Following the isolation of PBMCs, CD4+ T cells were selectively obtained through positive selection using magnetic beads covered with anti-CD4 antibodies (Miltenyi Biotec, Westphalia, Germany). The isolated CD4+ T cells were subsequently positively selected by magnetic beads coated with anti-CXCR5 antibodies to obtain CD4+CXCR5+ Tfh cells. The degree of purity of the sorted cells was evaluated through the application of flow cytometry.

Flow Cytometry

The proportions of cTfh and cTfr cells in CD4+CXCR5+ T cell cultures were analyzed using flow cytometry. The cells were treated with antibodies conjugated to fluorochromes targeting CD4, CXCR5, PD-1, ICOS (cTfh), and Foxp3 (cTfr) (Miltenyi Biotec, Westphalia, Germany) followed by the acquisition on FACSCalibur. The values observed in the individual quadrants indicated the proportion of cTfh and cTfr cells.

CCK-8 Assay

A CCK-8 kit (Abcam, Cambridge, UK) was performed to evaluate the toxicity of recombinant human PD-L1 protein on CD4+CXCR5+ T cells. Isolated cells treated with varying PD-L1 concentrations were cultured for 24 hours followed by the addition of CCK-8 reagent. Absorbance measurements were conducted at 450nm, and the rates of proliferation inhibition were subsequently computed.

ELISA

The concentration of IL-21 in supernatants of cultures was measured by a commercially available ELISA kit (Waltham, Massachusetts, United States). Supernatants collected from CD4+CXCR5+ T cell cultures treated with recombinant PD-L1 protein were added to pre-coated plates and color development was measured at 450nm.

Real-time PCR

The extraction of total RNA from CD4+CXCR5+ T cells was done by QIAGEN RNA extraction kit (Hilden, Germany) and mRNA expression of PI3K, AKT, Blimp-1, and Bcl-6 was assessed by two-step real-time PCR. GAPDH was used as an internal control and relative fold changes were determined utilizing the 2-ΔΔCt method.

Statistical Analysis

Statistical analysis was conducted using SPSS 17.0 and GraphPad Prism 5.0 software (CA Company, USA).

The normal distribution of the data was examined using the Kolmogorov-Smirnov test. In cases where the data exhibited a normal distribution, comparisons between two groups were assessed using an unpaired Student's t-test. Groups were compared using the one-way ANOVA followed by Tukey's post-hoc test. For data not following a normal distribution, non-parametric tests including the Mann-Whitney U test for two groups and the Kruskal-Wallis test for multiple groups were used. Changes were considered statistically significant when $p<0.05$.

RESULTS

Isolation of T Cells

Using the magnetic bead sorting method, first perform negative sorting CD4+T cells on PBMC, and then perform CXCR5 positive sorting; The purity of CD4+CXCR5+T and CD4+T cells obtained from sorting was determined, and the purity of CD4+T cells was greater than 93.7% (fluctuating between 93.7-99%); The CD4+CXCR5+T purity is greater than or equal to 80.7% (Figure 1).

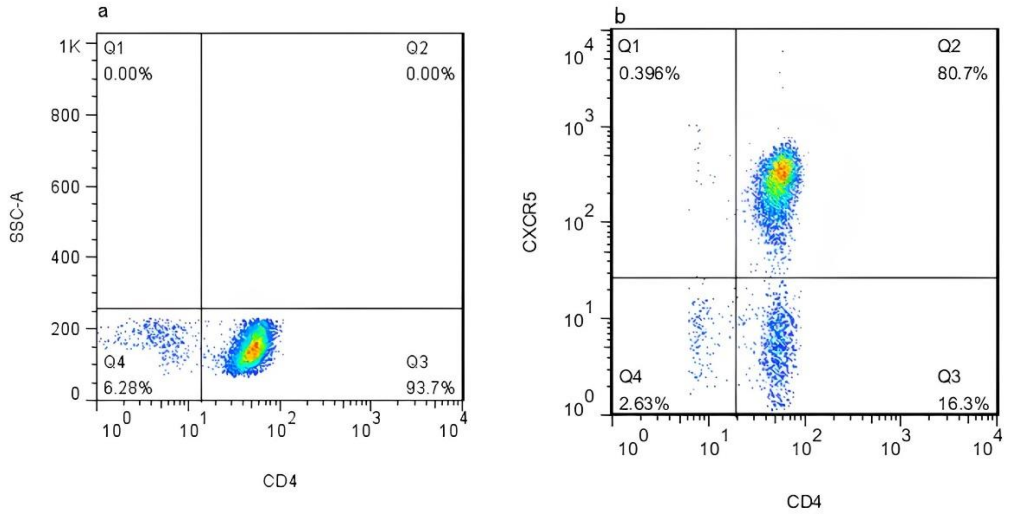

Figure 1. Flow cytometry of CD4+T cells and CD4+CXCR5+T purity after magnetic bead sorting. a. CD4+T purity: lower right quadrant; b. CD4+CXCR5+T purity: upper right quadrant.

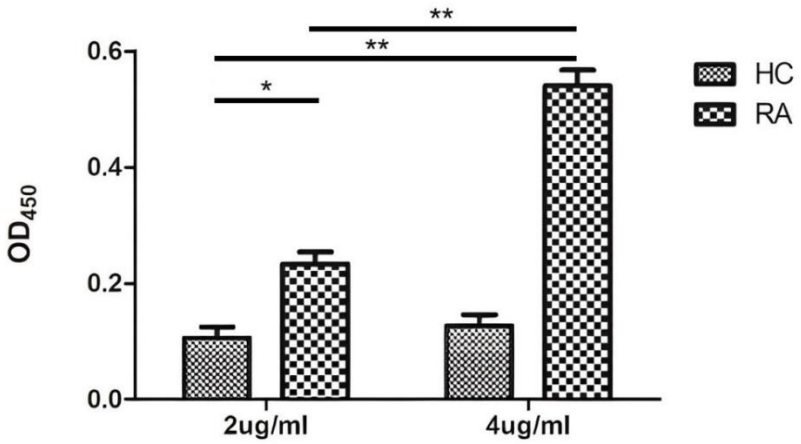

Figure 2. Cell counting kit 8 (CCK-8) detected the effect of varying doses of recombinant human programmed cell death 1 (PD-L1) protein on Rheumatoid arthritis (RA) and healthy controls (HC) peripheral blood CD4+CXCR5+T cell proliferation. RA CD4+CXCR5+T cell inhibition was concentration-dependent, with larger concentrations inhibiting more cells. Healthy persons were not inhibited. * $p<0.05$, ** $p<0.01$.

Recombinant Human PD-L1 Inhibits the Proliferation of CD4+CXCR5+T Cells in Patients with RA

Current work involved CD4+CXCR5+T cell isolation from the peripheral blood of individuals with RA and a healthy control group. These isolated cells were then subjected to in vitro culture, wherein varying amounts of recombinant human PD-L1 protein were added. The cells were further cultivated for 24 hours. The CCK-8 assay was employed to assess the growth inhibition rate. The findings indicated that the groups treated with low dose (2ug/mL) and high dose (4 ug/mL) of recombinant human PD-L1 protein exhibited a significantly higher inhibitory rate of CD4+CXCR5+T cells in the peripheral blood of individuals with rheumatoid arthritis in comparison with healthy controls (0.21±0.15 vs. 0.09±0.07, 0.42±0.20 vs. 0.14±0.09), with statistical significance. Moreover, the inhibitory effect of recombinant human PD-L1 protein on CD4+CXCR5+T cells in the peripheral blood of rheumatoid arthritis individuals was found to be dependent on the concentration. The inhibitory rate in CD4+CXCR5+T cells is directly proportional to of recombinant human PD-L1 protein concentration. However, it is worth noting that there was no statistically significant inhibition observed in healthy individuals, as depicted in Figure 2.

Changes in Peripheral Blood CD4+CXCR5+T Cells cTfh Treated with PD-L1 Protein in vitro

For examining the impact of recombinant human PD-L1on the frequency of cTfr, cTfh, and the ratio of cTfr/cTfh in the peripheral blood of individuals with RA, CD4+CXCR5+ T cells were isolated from the peripheral blood of both rheumatoid arthritis patients and healthy controls (HC). Following isolation, these cells were cultured in vitro and subsequently subjected to stimulation with various concentrations of recombinant human PD-L1 protein. No statistically significant findings were indicated in the proportion of cTfh within the overall population of cultured cells when comparing healthy CD4+CXCR5+T cells treated with varying amounts of recombinant human PD-L1 protein to the control group. The study showed a reduction in the proportion of cTfh cells between total cultured cells when treated with varying concentrations of recombinant human PD-L1 protein in patients with RA. Furthermore, no significant differences were found in the proportion of cTfh cells as the concentration of recombinant human PD-L1 protein increased. These findings are illustrated in Figure 3 and Figure 4.

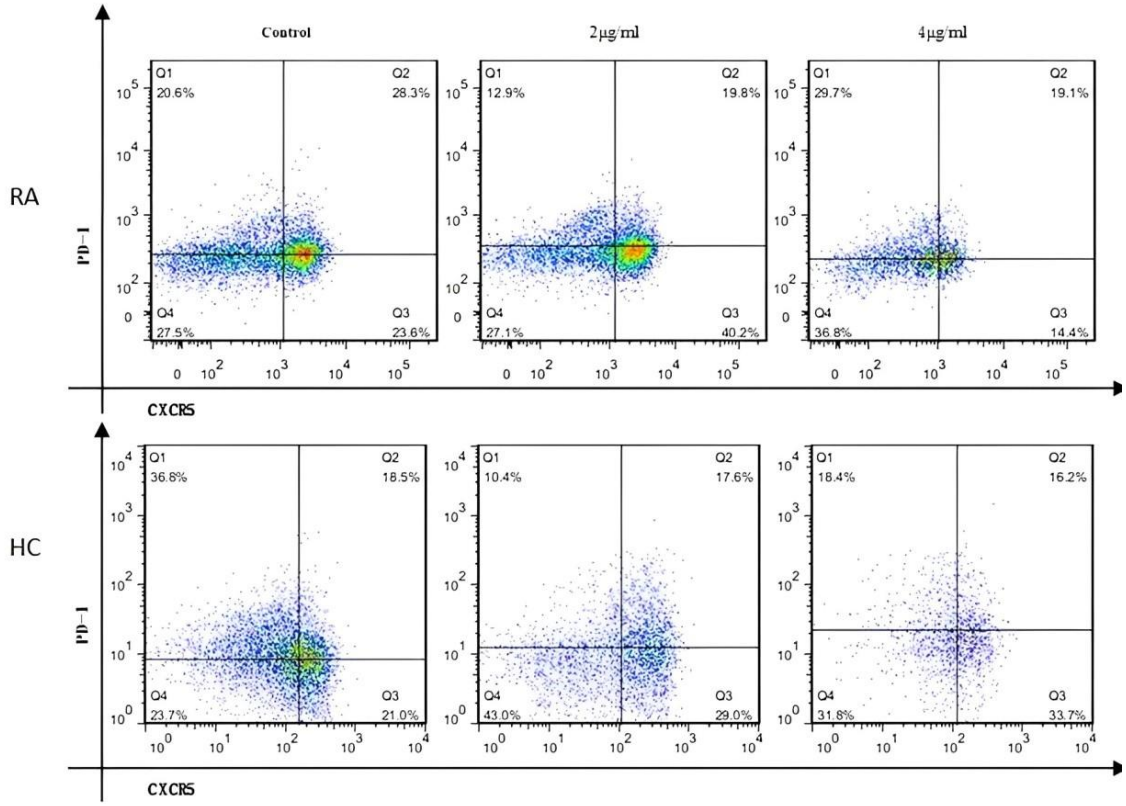

Figure 3. Cultured CD4+CXCR5+T cells isolated from the peripheral blood of controls and Rheumatoid arthritis (RA) patients in vitro. These cells were then stimulated with varying amounts of recombinant human programmed cell death 1 (PD-L1) protein. The purpose of this stimulation was to get circulating T follicular helper (cTfh) surface labeling using flow cytometry. The value in the top right quadrant represents the proportion of cTfh cells in the CD4+CXCR5+T cell population of individuals with RA and healthy controls (HC).

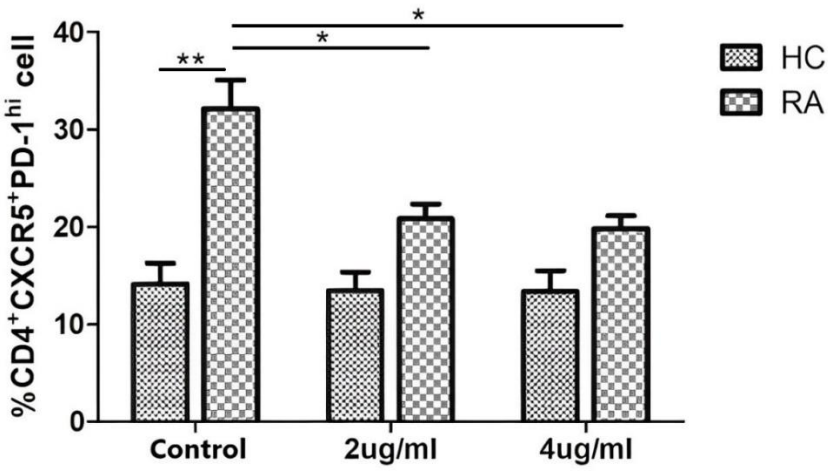

Figure 4. Changes in circulating T follicular helper (cTfh) to total CD4+CXCR5+T cell proportions of Rheumatoid arthritis (RA) and healthy controls (HC) after stimulation with different concentrations of recombinant human programmed cell death 1 (PD-L1). * p<0.05, ** p<0.01.

Changes in cTfr of CD4+CXCR5+T Cells Treated with Recombinant PD-L1 Protein in vitro

CD4+CXCR5+T cells of HC and RA patients were selected and cultured in vitro. Different concentrations of recombinant human PD-L1 protein were used to stimulate CD4+CXCR5+T cells. The findings indicated no statistically significant alteration in the proportion of cTfr within the overall population of cultured cells derived from healthy CD4+CXCR5+T cells, after treatment with various quantities of recombinant human PD-L1 protein. The observed proportion of cTfr in the overall population of cultured cells, which were subjected to varying concentrations of recombinant human PD-L1 protein, exhibited a declining pattern in CD4+CXCR5+T cells among RA individuals. However, statistical analysis revealed no significant disparity in comparison with the controls (Figures 5 and 6).

Changes in cTfr/cTfh Ratio of CD4+CXCR5+T Cells Administrated with Recombinant PD-L1 Protein in vitro

To examine the potential alteration of the cTfr/cTfh balance in CD4+CXCR5+T cells of individuals with RA following stimulation with recombinant human PD-L1 protein, we analyzed the cTfr/cTfh ratio within the stimulated cell population. The findings indicated a significant elevation in the cTfr/cTfh ratio of CD4+CXCR5+T cells in the peripheral blood of individuals with RA who were treated with varying quantities of recombinant human PD-L1 protein, in comparison with controls (p<0.05). cTfr/cTfh ratio of CD4+CXCR5+T cells in the peripheral blood of healthy individuals who were treated with varying quantities of recombinant human PD-L1 protein had no significant difference when compared to the control group (Figure 7).

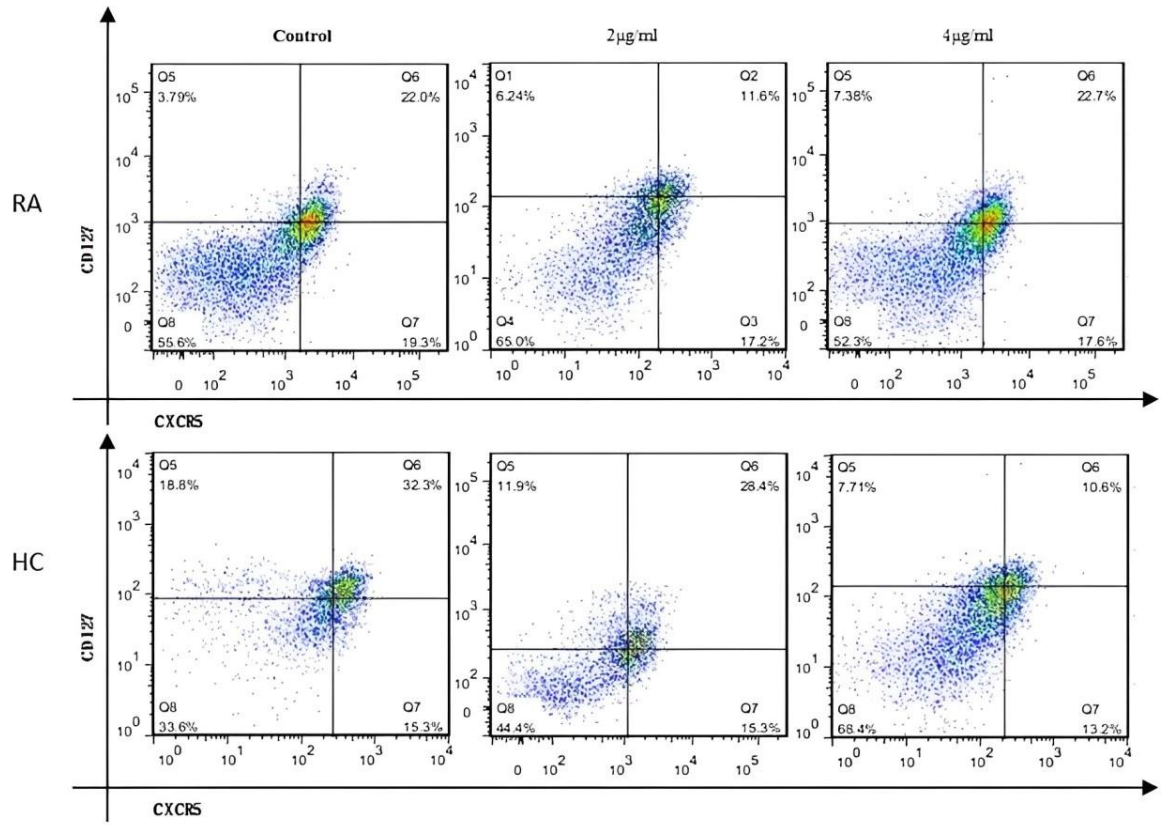

Figure 5. CD4+CXCR5+T cells of healthy controls (HC) and Rheumatoid arthritis (RA) were cultured and stimulated with various concentrations of recombinant human programmed cell death 1 (PD-L1) protein to obtain circulating T follicular regulatory (cTfr) surface staining flow cytometry. The lower right quadrant value is the cTfr percentage of CD4+CXCR5+T cells of RA and HC.

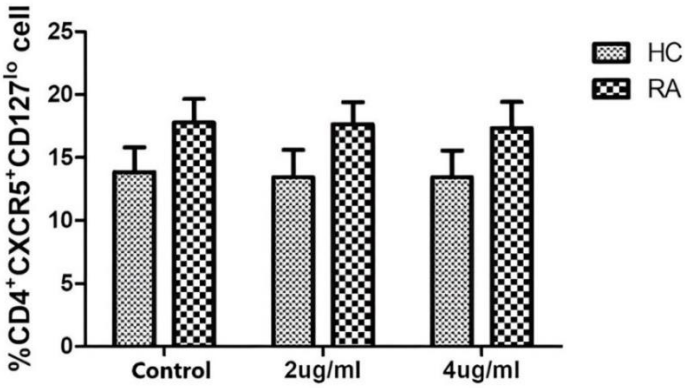

Figure 6. Rheumatoid arthritis (RA) and healthy controls (HC) peripheral blood circulating T follicular regulatory (cTfr) to total cell ratios in CD4+CXCR5+T cells following stimulus with different doses of recombinant human programmed cell death 1 (PD-L1) protein. A lowering trend in cTfr in peripheral blood CD4+CXCR5+T cells of RA patients treated with varying doses of recombinant human programmed cell death 1 (PD-L1) protein was not statistically significant. Among the healthy person treatment and control groups, there was no difference (p>0.05).

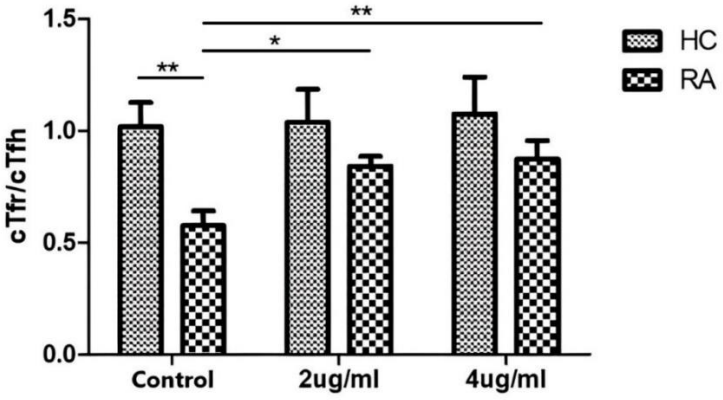

Figure 7. Changes in Rheumatoid arthritis (RA) and healthy controls (HC) CD4+CXCR5+T cell circulating T follicular regulatory (cTfr)/ circulating T follicular helper (cTfh) ratios following stimulation with various doses of recombinant human programmed cell death 1 (PD-L1) protein. The CD4+CXCR5+T cells of RA individuals treated with different quantities of recombinant human PD-L1 protein had a statistically significant higher cTfr/cTfh ratio. No significantly different data was observed between the treatment and control groups of healthy individuals. * p<0.05, ** p<0.01.

Effect of recombinant human PD-L1 protein on cytokines in the supernatant of CD4+CXCR5+T cells cultured in vitro in patients with RA

IL-21 is the main functional factor of Tfh. IL-21 promotes the differentiation of Tfh cells in a positive autocrine feedback circuit. Therefore, we will further investigate the concentration of IL-21 in the supernatant of the culture. Treatment with low-dose (2 ug/mL) and high-dose (4 ug/mL) recombinant human PD-L1 protein resulted in a lower IL-21 concentration in patients with RA compared to the control group (29.88±15.87 vs. 73.24±24.97, 46.20±20.82 vs. 73.24±24.97). The culture supernatant IL-21 concentration did not differ between healthy CD4+CXCR5+T cells administrated with varied doses of recombinant human PD-L1 protein and the control group. At the same time, we found that IL-21 in the culture supernatant of RA patients was higher in the control group and low-dose (2ug/ml) recombinant human PD-L1 protein group than in healthy individuals (73.24±24.97 vs. 32.13±10.22, 46.20±20.82 vs. 28.03±9.68), with statistical significance (Figure 8).

Recombinant PD-L1 Inhibits Blimp-1, Bcl-6, PI3K, and AKT mRNA Expression

RT-PCR was performed to evaluate the mRNA levels of Bcl-6 in CD4+CXCR5+T cells in RA and healthy individuals after the treatment of recombinant human PD-L1 protein at different concentrations. The experiments indicated that: (1) the relative expression of Bcl-6 mRNA in CD4+CXCR5+T cells at various doses of recombinant human PD-L1 in RA was lower, and no significant changes were observed in the relative mRNA expression of Bcl-6 in CD4+CXCR5+T cells inhibited by recombinant human PD-L1 protein at different concentrations (p>0.05). (2) In comparison to the controls, there was no difference in the relative Bcl-6 mRNA expression in CD4+CXCR5+T cells between the different dose groups of recombinant human PD-L1 in healthy individuals and controls (p>0.05). In comparison to the controls, the recombinant human PD-L1 protein significantly inhibited the Bcl-6 level in CD4+CXCR5+T cells of RA patients, but had no inhibitory effect on healthy individuals (Figure 9).

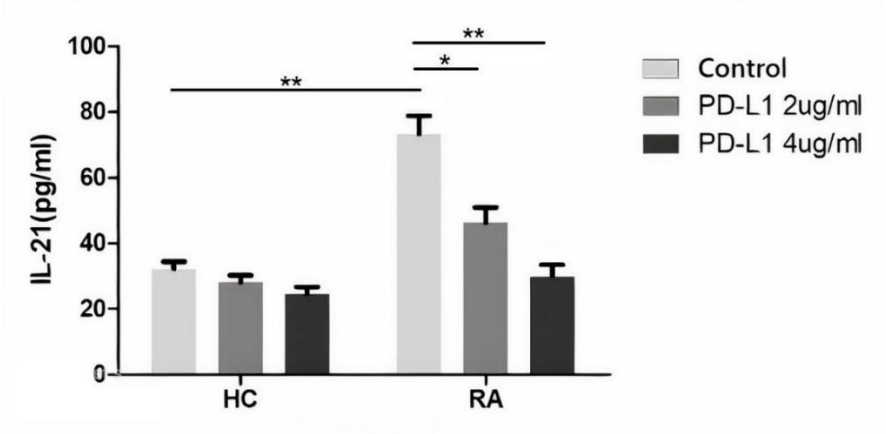

Figure 8. IL-21 concentrations in the culture supernatant of CD4+CXCR5+T cells in patients with Rheumatoid arthritis (RA) and healthy controls (HC) activated by recombinant human programmed cell death 1 (PD-L1) protein. In patients with RA, the CD4+CXCR5+T cells 2 ug/mL recombinant human PD-L1 protein treatment group had a significantly lower IL-21 content than the control group. * p<0.05, ** p<0.01.

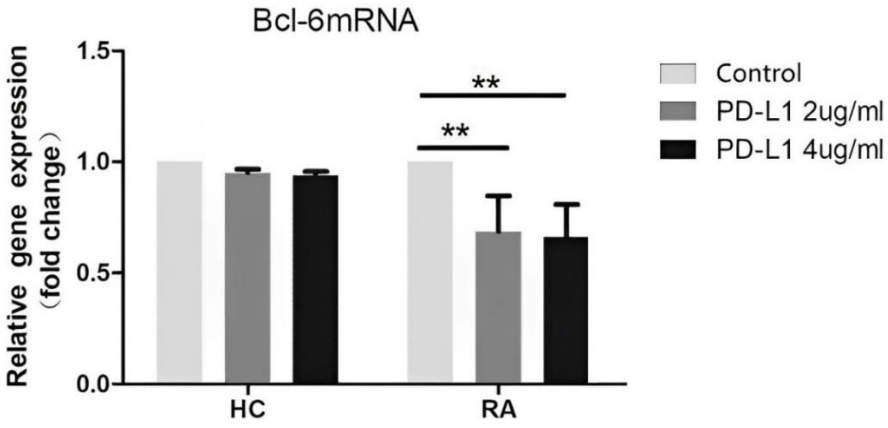

Figure 9. The expression level of Bcl-6 mRNA in CD4+CXCR5+T cells derived from the individuals with Rheumatoid arthritis (RA) and healthy controls (HC) treated with different concentrations of recombinant human programmed cell death 1 (PD-L1) protein was determined by employing the technique of reverse transcription polymerase chain reaction (RT-PCR); The data obtained from the experiment is presented as the mean value±the standard deviation. To assess the variances between the groups, statistical analyses such as t-tests or ANOVA are employed ** p<0.01.

RT-PCR was applied to determine the level of Blimp-1 mRNA in CD4+CXCR5+T cells of RA and healthy individuals after treatment with different concentrations of recombinant human PD-L1 protein. The experimental results showed no difference in the relative mRNA expression of Blinmp-1 in CD4+CXCR5+T cells (2 △ △ ct) among the groups of RA and healthy individuals with different doses of recombinant human PD-L1 protein and the control group (2 △ ct=-1) (n.s p>0.05) (Figure 10).

After treating CD4+CXCR5+T cells of RA and HC with different doses of recombinant human PD-L1 protein, RT-PCR was utilized to assess PI3K level. Recombinant human PD-L1 protein in RA resulted in lower expression of PI3K mRNA in CD4+CXCR5+T cells. No significant changes in mRNA levels at various concentrations (high-dose 4ug/ml, low-dose 2 ug/mL) were observed. In healthy persons, recombinant human PD-L1 protein treatment resulted in a lower mRNA level of PI3K in CD4+CXCR5+T cells compared to the control group.[3] In each dose group of human PD-L1 in RA, the relative mRNA expression of the signal transduction molecule PI3K decreased more than in healthy individuals. In conclusion, the recombinant human PD-L1 protein inhibited PI3K mRNA levels in CD4+CXCR5+T cells of patients with RA more than the control group (Figure 11).

After treating CD4+CXCR5+T cells of RA and healthy patients with recombinant human PD-L1 protein at different doses, RT-PCR was utilized to measure AKT levels. The study found that RA patients had lower mRNA expression of signal transduction molecule AKT in CD4+CXCR5+T cells at various doses of recombinant human PD-L1 protein compared to the control group. However, no significant variation was observed in the mRNA levels of AKT in CD4+CXCR5+T cells inhibited by different concentrations (p>0.05). Recombinant human PD-L1 protein in healthy individuals resulted in reduced mRNA expression of the signal transduction molecule AKT in CD4+CXCR5+T cells in comparison with the control group. In rheumatoid arthritis patients, the administration of recombinant human PD-L1 protein resulted in a significant reduction in AKT mRNA expression at both doses, with the decrease observed being more substantial compared to that in healthy individuals. In conclusion, the recombinant human PD-L1 protein Inhibited AKT mRNA in CD4+CXCR5+T cells of RA individuals more than the control group (Figure 12).

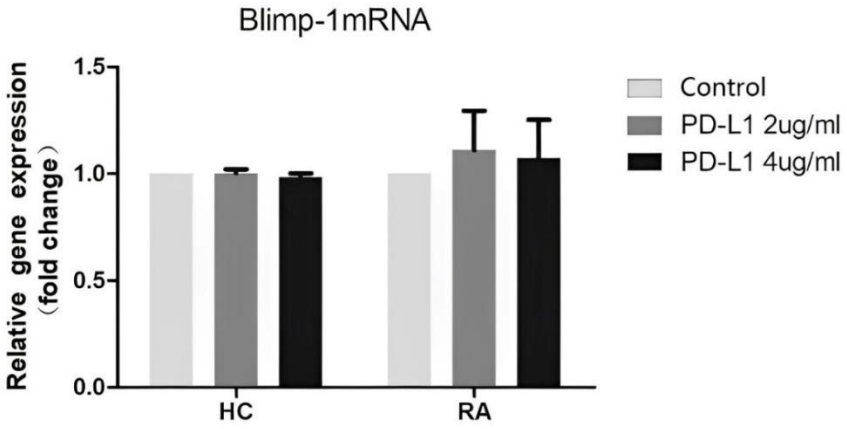

Figure 10. RT-PCR was performed to evaluate the level of Blinmp-1 mRNA in peripheral blood CD4+CXCR5+T cells of Rheumatoid arthritis (RA) and healthy controls (HC) treated with different concentrations of recombinant human programmed cell death 1 (PD-L1) protein. The data obtained from the experiment is presented as the mean value±the standard deviation. To assess the variability between the groups, statistical analyses such as t-tests or ANOVA are employed.

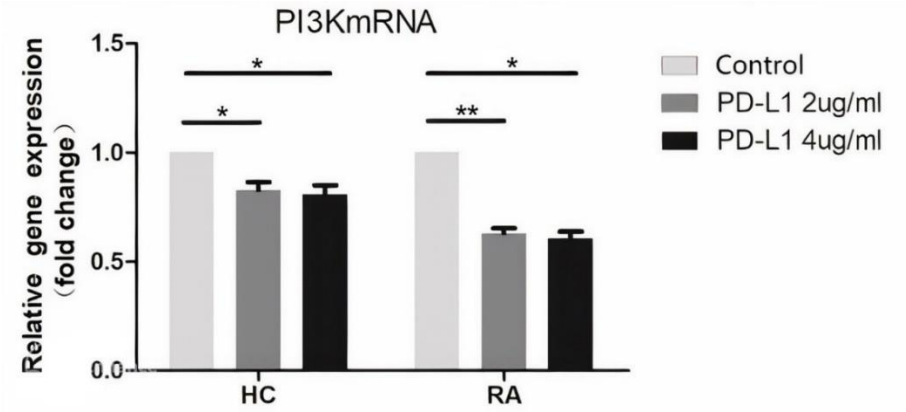

Figure 11. RT-PCR was performed to measure the level of PI3K in CD4+CXCR5+T cells from Rheumatoid arthritis (RA) and healthy controls (HC) treated with recombinant human programmed cell death 1 (PD-L1) protein at different concentrations. The data obtained from the experiment is presented as the mean value ± the standard deviation. To assess the variability between the groups, statistical analyses such as t-tests or ANOVA are employed. * p<0.05, ** p<0.01.

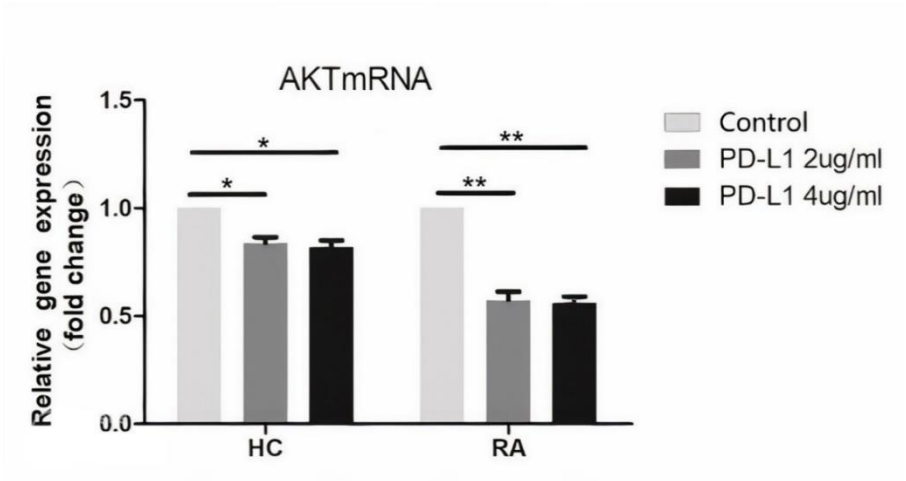

Figure 12. RT-PCR was performed to measure the level of AKT in CD4+CXCR5+T cells of Rheumatoid arthritis (RA) and healthy controls (HC) treated with recombinant human programmed cell death 1 (PD-L1) protein at different concentrations. The data obtained from the experiment is presented as the mean value ± the standard deviation. To assess the variability between the groups, statistical analyses such as t-tests or ANOVA are employed. * p<0.05, ** p<0.01.

DISCUSSION

Our study indicated that the recombinant PD-L1 protein effectively suppresses the proliferation of CD4+CXCR5+T cells, particularly cTfh cells, in individuals diagnosed with RA. This study is the initial investigation into the impact of recombinant human PD-L1on CD4+CXCR5+T cells in individuals diagnosed with RA, as far as current knowledge indicates. Significantly, the PD-L1 can restore the immunological imbalance between cTfr and cTfh in RA individuals.

Prior research has demonstrated that there is an elevation in circulating Tfh cells among individuals diagnosed with RA, but Tfr cells either decrease or exhibit little alterations. Consequently, there is a disruption in the equilibrium of Tfh/Tfr cells.[17] It aligns with the outcomes of our investigation. As the condition of individuals with RA improves, there is a drop in cTfh and an increase in Tfr. These changes are inversely connected with RA, which helps to limit the autoimmune response in RA patients and stabilize their condition.[8,18] Hence, there is speculation on the potential

efficacy of modulating the immunological equilibrium between Tfr cells and Tfh cells for the clinical management of RA. The control of the cytokine microenvironment has a crucial role in determining the immunological balance between Tfh cells and Tfr cells. The RA patients exhibited a detectable upregulation of IL-21, IL-6, and sPD-1 expression. Upregulation of interleukin-21 (IL-21) has been shown to have inhibitory effects on regulatory T follicular (Tfr) cells, while also promoting their differentiation.[8,19.]

The PD-L1/PD-1 signaling pathway plays a crucial role in promoting peripheral immunological tolerance and holds great significance in the treatment of autoimmune disorders. Studies have demonstrated that the interaction between PD-L1 and its receptor PD-1 actively triggers the development of regulatory T cells (Treg) and boosts their suppressive capabilities, while simultaneously suppressing the response of effector T cells.[11,20] The available data provides evidence that PD-1 acts as a facilitator in developing Tfr cells. A study [23] observed a reduction in the quantity of Tfr cells within the lymph nodes of mice lacking in PD-L1/PD-1. PD-1 may facilitate the contact between homologous T-B cells and transfer inhibitory signals to Tfh cells.[7,21] Blocking the interaction between PD-1 and its ligands has been shown to result in an upregulation of Tfh differentiation.[10] This suggests that PD-1 has a negative regulatory impact on the differentiation of cTfh cells. The inhibition of Tfh cells is observed with the administration of PD-1.[22] The inhibition of PD-1 resulted in a decrease in the production of cytokines associated with Tfh cells, hence inhibiting the production of plasma cells.[10] This work reveals that the recombinant human PD-L1 protein effectively hinders the development of Tfh cells, diminishes the proportion of Tfh cells, and decreases the expression of IL-21, which serves as the primary functional factor of Tfh cells. Consequently, the Tfr/Tfh ratio has a tendency to approach typical proportions in the human population. The findings propose that the modulation of Tfr/Tfh cells via the PD-L1/PD-1 pathway has promise as a possible therapeutic approach for those afflicted with RA.

The transcription factor Bcl-6 has an important impact on the process of Tfh cell development. The Bcl-6 level can be promoted by the production of IL-6 and IL-21.[19] Bcl-6 has the ability to stimulate the CXCR5 level, convert non-Tfh cells into Tfh cells, and govern the process of Tfh differentiation and development.[23] The results of our investigation indicate that the recombinant PD-L1 protein effectively suppresses the release of IL-21 and diminishes the level of Bcl-6 mRNA. Bcl-6 can exert inhibitory effects on Blimp-1, a protein that functions as an inhibitor of Bcl-6. Blinmp-1 has been observed to exhibit the ability to downregulate the Bcl-6 level in CD4+T cells, as reported in a previous study.[21] Our data indicated that there was no statistically significant alteration observed in the level of Blimp-1 mRNA in CD4+CXCR5+T cells following treatment with recombinant PD-L1 protein. These results propose that PD-L1 protein treatment may not have a substantial impact on Tfh transcription, where Blimp-1 mRNA is believed to have a crucial role.

The communication between PD-1, expressed on T lymphocytes, and PD-L1 triggers co-stimulatory signals that result in the phosphorylation of tyrosine residues within the ITSM domain of PD-1. Consequently, downstream protein kinases Syk and PI3K undergo dephosphorylation, leading to the inhibition of downstream pathways such as AKT and ERK. This inhibition ultimately hampers T lymphocyte proliferation, differentiation, and cytokine secretion, thereby damaging the action of CD8+T lymphocytes and CD4+T lymphocytes. Our present study successfully identified PI3K and AKT levels and observed a marked drop in the levels of both molecules. The present study conducted an initial investigation into the regulatory impact of the PD-L1/PD-1 signaling pathway via the PI3K/AKT cascade.

The PD-L1/PD-1 interaction is known to inhibit cascades such as the PI3K/Akt pathway,[24] as was demonstrated in our study. However, the exact regulatory effect of PD-L1 on the balance between cTfr and cTfh cells deserves further exploration. For example, it would be informative to examine the phosphorylation status of key nodes in the PI3K/Akt pathway like PDK1, GSK3β, and mTOR following PD-L1 stimulation. Epigenetic modifiers and transcription factors downstream of this cascade that modulate the cTfr/cTfh balance could also be investigated. Considering that our study only assessed the peripheral immune response, further research should be conducted to determine the effects of PD-L1 on Tfr and Thf cells in lymph node germinal centers. Techniques like immunohistochemistry and fluorescence immunostaining of lymph node sections from RA patients could provide insight into the spatial dynamics of these cell subsets. Animal models of autoimmunity recapitulating the cTfr/cTfh imbalance in RA, such as the CIA mouse, could also be used to test PD-L1 therapy and tease out tissue-specific responses. Single-cell RNA sequencing of

lymph node cells before and after treatment may help delineate novel cell states and pathways targeted by PD-L1. Finally, considering the translatability of these findings, clinical studies evaluating PD-L1 blockade either as monotherapy or in combination with other agents are warranted for RA.

This research elucidates the impact and potential processes of recombinant human PD-L1 protein on cTfh cells, cTfr cells, and their equilibrium. The findings of our investigation indicate that the administration of recombinant human PD-L1 protein effectively restores the equilibrium of the cTfr/cTfh ratio in RA individuals. The PD-L1/PD-1 cascade has inhibitory impacts on the proliferation, differentiation, and activation of cTfh cells by modulating the PI3K/AKT cascade. This regulatory mechanism plays a crucial role in maintaining immunological homeostasis in conjunction with cTfr cells. Hence, the augmentation of the communication between PD-L1/PD-1 holds potential as a viable therapeutic approach for individuals with RA.

STATEMENT OF ETHICS

The Medical Ethics Committee of the First Affiliated Hospital of Jinzhou Medical University approved this study. Also, the Science and Technology Department of Liaoning Province supported this study (ID:2019.01-2024.08). All RA and healthy volunteers signed informed consent forms.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Not applicable

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