ORIGINAL RESEARCH

# Inflammatory Cytokine-Neutralizing Antibody Treatment Prevented Increases in Follicular Helper T Cells and Follicular Regulatory T Cells in a Mouse Model of Arthritis

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**Background:** Follicular T helper (TFH) and follicular regulatory T (TFR) cells play important roles in humoral immunity. Nevertheless, their significance in rheumatoid arthritis (RA) pathogenesis has not been fully elucidated. As an important treatment strategy, the effect of inflammatory factor-neutralizing antibodies on TFH and TFR in RA remains unclear.
**Methods:** We used the collagen-induced arthritis (CIA) mouse model to illustrate the quantity and functional changes in TFH and TFR cells. The changes of plasmablast, TFH and TFR cells in the spleen and peripheral blood of CIA mice were analyzed by flow cytometry. The levels of TFH and TFR and their functional subsets in the spleen after anti-inflammatory antibody treatment were analyzed and compared. The functional changes of TFH and TFR in CIA mice before and after treatment were detected by in vitro culture experiments.
**Results:** Plasmablast levels were increased in CIA spleen and peripheral blood and both TFH and TFR cell levels were upregulated. TFH and TFR cells were decreased significantly after the anti-inflammatory antibody treatment. TIGIT$^+$ and TIGIT$^+$CD226$^-$ TFH cells in CIA mouse spleen were elevated and PD-1 and ICOS expression on spleen TFH and TFR cells was increased. Both the ability of TFH cells to secrete IL-21 and aid B cells and the ability of TFR cells to secrete IL-10 and inhibit TFH cells were enhanced in the CIA mice. After antibody treatment, the cell subsets and functions were recovered.
**Conclusion:** Germinal center TFH and TFR cells were increased and their functions were enhanced. With inflammatory factor-neutralizing antibody treatment, TFH and TFR subsets and their functions returned to normal. These findings provide important information on the dynamics of humoral immune-related cell subsets in RA and the effects of treatment on them.
**Keywords:** TFH, TFR, collagen-induced arthritis, rheumatoid arthritis, inflammatory cytokines

## Introduction

Rheumatoid arthritis (RA) is a clinically common chronic autoimmune disease involving symmetric peripheral multi-joint disease. Its main pathological manifestations include inflammatory cell infiltration, angiogenesis, and joint cartilage destruction, which subsequently leads to joint malformations or even functional loss.[1,2] A feature of RA is the production of a variety of autoantibodies.[3] Pathogenic autoantibodies caused by abnormal T and B lymphocyte function are important factors involved in RA pathogenesis.[4] However, the detailed pathogenesis of RA has not been fully clarified and the relevant regulatory mechanism remains unclear.

CD4$^+$ T cells play very important roles in RA pathogenesis. Helper T (TH)17 cells and regulatory T cells (Treg) have opposite roles in the autoimmune and inflammatory responses.[5–7] In recent years, an increasing number of studies have

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shown that a specific CD4^{+} T cell subset, follicular TH (TFH) cells, plays a key role in humoral immunity.[8–10] TFH cells are important for germinal center formation and humoral responses and secrete IL-21 and aid B cells by supporting class-switching, somatic hypermutation, and high-affinity autobody production. The role of TFH cells in autoimmune diseases has received increasing attention.[4,11–15] TFH cells are increased in autoimmune diseases and play a disease-promoting role in autoimmune diseases.[4,11–15] Increased numbers of peripheral blood TFH-like cells have been detected in patients with primary Sjögren's syndrome, dermatomyositis, and systemic lupus erythematosus (SLE) and can produce large amounts of autoantibodies.[11–13] The numbers of circulating CD4^{+}CXCR5^{+}, CD4^{+}CXCR5^{+}PD1^{+}, and CD4^{+}CXCR5^{+}PD1^{hi} TFH cell subsets were increased in RA.[4,14,15]

Follicular regulatory T (TFR) cells can suppress TFH cell-mediated humoral immunity. TFR cells are a specific subset of Tregs expressing CXCR5, which has been widely studied in recent years.[16–21] TFR cells express FoxP3, Bcl-6, and Blimp-1 and inhibit TFH cells and germinal center responses.[16] Animal experiments have demonstrated that the lack of TFR cells led to a larger germinal center reaction in vivo,[17] and TFR conditional knockout promoted autoimmune disease in mice.[18] Currently, there is ongoing research on the role of TFR cells in autoimmune diseases. Compared with healthy controls, the peripheral blood of patients with ankylosing spondylitis contained a significantly increased frequency of CD4^{+}CXCR5^{+}FOXP3^{+} TFR cells.[19] Our group reported significantly increased CD4^{+}CXCR5^{+}FOXP3^{+} TFR cells in the peripheral blood of patients with SLE as compared with healthy controls.[20] We have reported that patients with RA in stable remission had significantly increased levels of circulating CD4^{+}CXCR5^{+}FoxP3^{+} TFR cells and that the TFR/TFH ratio was significantly increased in patients with RA in stable remission.[21] TFR cells were negatively correlated with disease activity score 28 (DAS28), which reflect the condition of RA.[21]

A series of surface markers are important for differentiating the T cell subsets, and include TIGIT, CD226, PD-1, and ICOS. TIGIT is a newly discovered co-suppressive molecule on T cells that exerts immunosuppressive effects by competing with CD226 for the same CD155 ligand.[22] TIGIT^{+} TFH cells have stronger ability to aid B cells.[23] Attenuating CD226 signals increased CXCR5, ICOS, and IL-21 expression by CD155-stimulated germinal center TFH cells.[24] PD-1–PD-L1 sends important signals to B cells, so high PD-1 expression on TFH cells is critical for B cell responses and antibody production.[25] ICOS is highly expressed on TFH cells and is an effective functional molecule on TFH cells to aid B cells.[26] ICOS deficiency results in the depletion of memory B cells that prevents antigen-specific IgG responses.[27] Therefore, ICOS and PD-1 expression represent activated TFH cells that play critical roles in helper B cell differentiation and antibody production.

The collagen-induced arthritis (CIA) model is one of the most common autoimmune models of RA. It has been widely used for identifying the potential pathogenic mechanisms of autoimmunity in RA, including the role of various cell types in RA pathogenesis and progression.[28,29] The CIA model also plays an important role in the testing and development of new biology-based treatments for autoimmune diseases.[28,29]

The changes in TFH and TFR cell expression and function during RA treatment have also attracted research attention. One RA treatment is to use neutralizing antibodies against inflammatory cytokines. Clinically, inflammatory factor-related neutralizing antibody drugs have been used in RA treatment, and include infliximab (monoclonal anti-TNFα antibody) and canakinumab (monoclonal anti-IL-1β antibody).[30–33] The inflammatory cytokine TNFα leads to the pathogenesis of various rheumatic diseases and is produced by innate and adaptive immune system cells.[30] Patients with RA often have high TNFα levels in the synovial fluid and synovial tissues, and TNFα inhibitors are effective for treating RA.[30,31] Canakinumab demonstrates significant efficacy in juvenile RA.[32] In patients with active RA, adding canakinumab to methotrexate treatment improved the treatment effect.[33] Inflammatory factor-neutralizing antibodies have been used in a series of studies on CIA models, and some studies have also used anti-TNFα and anti-IL-1 simultaneously.[34–36] Related studies have made important theoretical preparations for using these neutralizing antibodies to treat patients with RA. Although these antibodies had been used for treating CIA mice, how they affect TFH and TFR cells remains unclear.

In this study, we used the CIA mouse model to study the expression and functional changes of TFH and TFR cell subsets in the spleen germinal center in RA in-depth. Based on this, we explored the effect of inflammatory factor-neutralizing antibody treatment on TFH and TFR cell expression and function in vivo. This study will help deepen understanding of TFH and TFR cell expression and functional changes in RA disease and treatment.

## Materials and Methods

### Mice

Studies were conducted using 10-week-old female DBA/1 mice from Charles River Laboratories (Beijing, China). All mouse research protocols complied with Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines and were approved by the Peking University People's Hospital ethical board. The mouse research was performed in accordance with the Laboratory Guidelines for Animal Use and Care.[37]

### Induction and Assessment of CIA

Bovine type II collagen (CII; Chondrex, Redmond, WA, USA) was emulsified in Freund's complete adjuvant (FCA; Sigma-Aldrich, St. Louis, MO, USA) in equal volumes. Virgin female DBA/1 mice (10-week-old) kept in separate cages after weaning were intradermally immunized at the tail base with 100 μL emulsion (150 μg bovine CII). After 21 days, the mice received a second intradermal immunization with 150 μg CII emulsified in Freund's incomplete adjuvant (FIA; Sigma-Aldrich). CIA severity was determined by assessing the overall inflammation in individual paws. Each paw was graded as follows: 0, normal; 1, swelling of one finger; 2, swelling of >1 finger; 3, swelling in the entire paw; or 4, deformity and/or ankylosis. The total clinical score was the sum of these four scores and the maximum clinical score for each mouse was 16. To analyze TFH and TFR cell changes during CIA, the CIA mice were sacrificed at 5 weeks (w), 7w, 10w, and 13w after the first injection to detect the levels of the relevant subsets and compared with non-CIA healthy control age-matched mice in which the modeling treatment had not been applied. Four pairs of CIA and control mice were analyzed for each timepoint.

In terms of treatment experiments, additional CIA mice were established and two common treatment strategies for CIA mice were applied. At the 8w timepoint, these mice were randomized to three groups and injected intraperitoneally with anti-mouse/rat IL-1β (clone number B122), anti-mouse TNFα (clone number XT3.11)+anti-mouse/rat IL-1β (clone number B122), or isotype IgG (as the control) three times a week, and the total amount of three consecutive injections was 0.9 mg per antibody. The treatment antibodies were purchased from Bio X Cell (West Lebanon, NH, USA). The three groups (n = 4 mice per group) were sacrificed at 10w, and TFH and TFR cells from the spleen and peripheral blood were analyzed by flow cytometry.

### Flow Cytometry and Intracellular Cytokine Detection

Mouse spleens were acquired and ground into cell suspensions and the lymphocytes were isolated in separation medium (Dakewei Biotech Co., Ltd., Shenzhen, China). The lymphocytes were stained for 30 min in PBS with antibodies against surface markers, which included anti-CD4-PE-Cy7, anti-CXCR5-Alexa Fluor 647, anti-PD-1-APC-Cy7, anti-ICOS-FITC, anti-TIGIT-PE, and anti-CD226-PE-Cy7 (BioLegend, San Diego, CA, USA). The cells were washed twice with PBS and intracellular staining was performed with a FoxP3/Transcription Factor Staining Buffer Kit (TONBO Biosciences, San Diego, CA, USA) according to the manufacturer's instructions. After fixation and permeabilization, anti-FoxP3-PerCP-eFluor710 (eBioscience, Thermo Fisher Scientific, San Diego, CA, USA) was added and incubated for 30 min. For B cell analysis, anti-GL7-Alexa Fluor 647, anti-B220-APC-Cy7, and anti-CD13- PE-Cy7 (BioLegend) were surface-stained, then intracellular staining was performed using anti-IgG1-FITC (BioLegend). Germinal center $B220^+IgG1^+GL7^+$ cells and $B220^+CD138^+IgG1^+$ plasmablasts were analyzed and compared between the CIA and control mice.

To detect cytokine production by the lymphocytes, the cells were cultured with phorbol 12-myristate 12-acetate (PMA, 50 ng/mL) and ionomycin (1 μg/mL) (PeproTech, Rocky Hill, NJ, USA) for 5 h with brefeldin A (1000×, BioLegend) in RPMI 1640 medium containing 10% fetal calf serum. After staining for surface markers, ie, anti-CD4-APC-Cy7 and anti-CXCR5-Alexa Fluor 647 (BioLegend), the cells were fixed and permeabilized, and incubated with the following antibodies: anti-FoxP3-PerCP-eFluor710 (eBioscience, Thermo Fisher Scientific), anti-IFN-γ-PE-Cy7, anti-IL-17A-FITC, anti-IL-21-PE, anti-IL-10-PE, anti-IL-4-PE-Cy7, and anti-TNFα-FITC (BioLegend). The samples were analyzed on a FACSCanto system using Diva software (BD, Biosciences, San Jose, CA, USA).

## In vitro Function Assay

Spleen $CD4^+CXCR5^+CD19^-GITR^-$ TFH cells ($5 \times 10^4$) from 10w CIA and control mice were plated with $5\times10^4$ $CD19^+$ B cells (from CIA mouse spleen) and anti-CD3 (2 μg/mL) plus anti-IgM (5 μg/mL) for 5 days in RPMI 1640 medium containing 10% fetal bovine serum and cultured at 37°C and 5% $CO_2$. For the TFR cell suppression assays, $1.5\times10^4$ spleen $CD4^+CXCR5^+CD19^-GITR^+$ TFR cells from 10w CIA and control mice were added to the wells along with TFH and B cells (both from CIA mouse spleen). All cells were sorted by a FACSAria II flow cytometer (BD Biosciences) and the purity of each cell subset was verified to be >97%. The cells and supernatant were harvested and analyzed after 5 days. $CD19^+$ B cells were surface-stained for GL7 and intracellularly stained for IgG1. IgG concentrations in the supernatant were measured by enzyme-linked immunosorbent assay (MabTech, Nacka Strand, Sweden). The fluorescent antibodies used for the in vitro functional assays were from BioLegend.

## Statistical Analysis

The results between the two groups were compared using Student's $t$ test. Non-normally distributed results were compared using the Mann–Whitney test. Comparisons between multiple groups were performed using one-way analysis of variance (ANOVA) and multiple comparisons. All analyses were carried out using Prism (GraphPad Software, San Diego, CA, USA). P-values < 0.05 were considered significant.

# Results

## Plasmablast Levels in the Spleen and Peripheral Blood of CIA Mice Were Significantly Upregulated

The CIA model was established in DBA/1 mice by two rounds of immunization with CII at 0w and 3w. The mice were dynamically observed and scored until 13w (Figure 1A and B). We selected the 10w peak disease timepoint to detect the B cell-related subsets in the spleen and peripheral blood. Based on the clinical scores and arthritis symptoms in the feet, the model was established successfully (Figure 1B and C). Analysis of the splenic lymphocytes to clarify changes in the germinal center B cells and plasmablasts revealed that the level of splenic germinal center $B220^+IgG1^+GL7^+$ cells from the CIA mice was significantly higher than that in the controls (Figure 1D and E). Analysis of the percentages of $B220^+$ $CD138^+$ $IgG1^+$ plasmablasts revealed that they were significantly upregulated in spleen and peripheral blood of the CIA mice (Figure 1F and G). These results indicate that there is greater B cell activation in the spleen and peripheral blood of CIA mice and that the ability of B cells to secrete IgG1 was significantly enhanced.

## TFH and TFR Cells in the Spleen Germinal Center and Peripheral Blood Gradually Increased in the CIA Mouse Model

We investigated the changes in two critical cell subsets related to B cell maturation: TFH and TFR cells. The levels of $CD4^+CXCR5^+FoxP3^-$ TFH and $CD4^+CXCR5^+FoxP3^+$ TFR cells in the spleen and peripheral blood of the CIA and control mice were analyzed at 5w, 7w, 10w, and 13w after the first immunization (0w). At each timepoint, four pairs of CIA and age-matched control mice were sacrificed and the spleen and peripheral blood lymphocytes were obtained for flow cytometry analysis. At the early stage of modeling (5w), the spleen TFH cell levels in the CIA mice and the controls were not significantly different. However, over time, both the percentage and absolute numbers of spleen TFH cells were significantly higher in the CIA mice than in the controls (Figure 2A and B). In the initial stage of modeling, there was little difference in the TFR cell levels, but over time, the TFR levels in the CIA mouse spleen were significantly higher than that of the control (Figure 2A and B). In the peripheral blood, the changes in the TFH and TFR cell levels were in accordance with that for the spleen (Figure 2C). Comparison of the TFH/TFR ratios revealed that there was no significant difference between the CIA mice and the controls (Figure 2D). These results suggested that TFH and TFR cell levels gradually increased in the germinal center during the RA process and that the changes in peripheral blood and germinal center TFH and TFR cells were consistent.
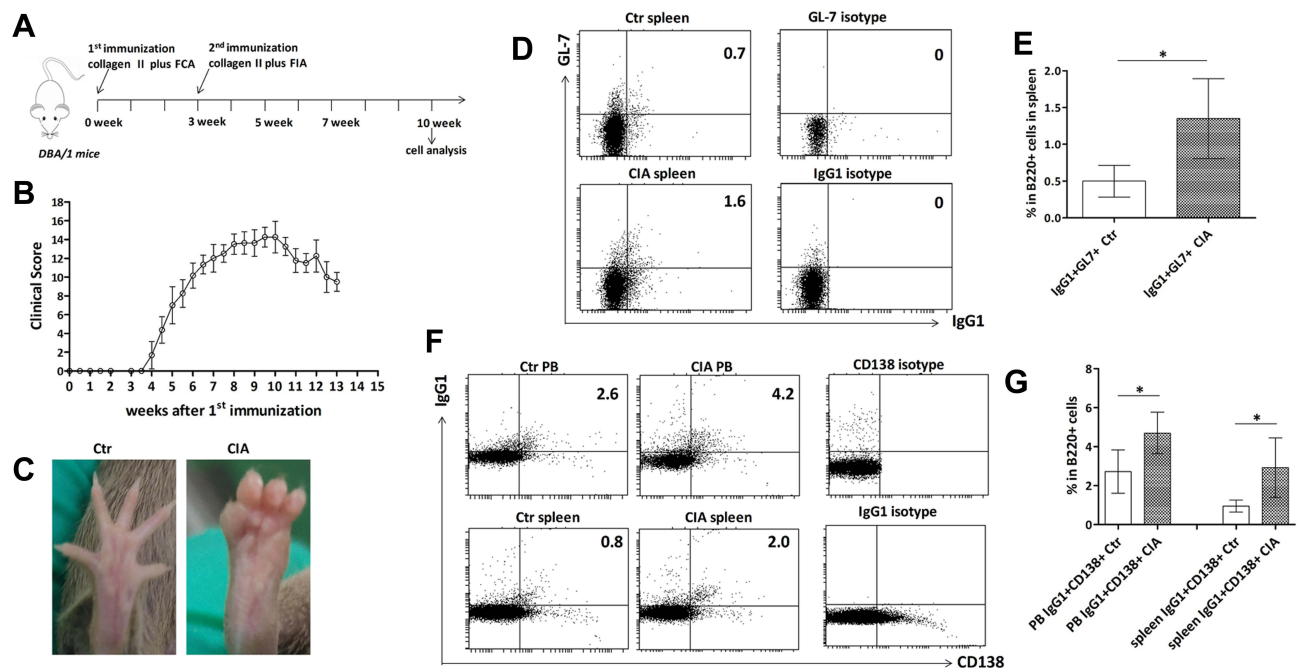
**Figure 1** B cell subset analysis of CIA mouse spleen and peripheral blood. (**A**) The CIA mouse model was established using 10-week-old DBA/1 mice, which underwent two cycles of immunizations (at 0w and 3w). Healthy age-matched DBA/1 mice were used as the Ctr group. (**B** and **C**) The dynamic changes of arthritis scores in the CIA mouse model and pictures of foot arthritis in Ctr and CIA mice at 10w. (**D** and **E**) Comparison of the IgG1$^+$GL7$^+$ germinal center B cell in B220$^+$ cells in the spleen of four pairs of Ctr and CIA mice (at 10w). (**F** and **G**) Comparison of the CD138$^+$IgG1$^+$ plasmablast percentages in B220$^+$ cells in the peripheral blood (PB) and spleen of four pairs of Ctr and CIA mice. Data are from a single experiment representative of two independent experiments. Gating was according to isotype controls. Data are the mean ± SD. Results were compared using Student's $t$ test. *P < 0.05.
**Abbreviations**: CIA, Collagen-induced arthritis; Ctr, control; PB, peripheral blood; SD, standard deviation.

## TFH and TFR Cells in Antibody-Treated CIA Mouse Spleen and Peripheral Blood Were Significantly Reduced

We performed additional experiments using neutralizing antibodies against inflammatory cytokines for treating CIA. We selected two common CIA mouse treatment strategies to study the effects of inflammatory factor-neutralizing antibody (anti-TNFα+anti-IL-1β and anti-IL-1β alone) treatment on germinal center and peripheral blood TFH and TFR cells. At 8w, the CIA mice were randomized into three groups and injected intraperitoneally with anti-IL-1β, anti-TNFα+anti-IL-1β, or isotype IgG (control) three times a week (Figure 3A). We chose 10w as the termination timepoint and recorded significantly decreased clinical scores after antibody treatment, and the symptoms of arthritis were significantly relieved (Figure 3B and C). There were no significant differences between the mice injected with anti-TNFα+anti-IL-1β or anti-IL-1β alone (Figure 3B and C). After treatment with these two regimens, both spleen TFH and TFR cells exhibited a significant downward trend and peripheral blood TFH and TFR cells were also decreased significantly (Figure 3D and E). However, there was no significant difference between the two treatment options. Comparison of the TFH/TFR ratios revealed no significant difference among the three groups (Figure 3F). The results showed that TFH and TFR cells were significantly reduced in the antibody-treated CIA mice, suggesting that the result of anti-inflammatory immunotherapy is a persistent decrease in the mouse TFH/TFR system.

## TIGIT$^+$ and TIGIT$^+$CD226$^-$ TFH Cells Were Significantly Elevated in CIA Mouse Spleen and Their Levels Returned to Normal After Antibody Treatment

The main functionally relevant markers were analyzed to clarify the TFH and TFR subpopulation changes in the germinal center and peripheral blood of the CIA mice. The TIGIT$^+$ proportions in the CIA mouse spleen TFH cells were significantly higher than those of the control (Supplementary Figure S1A and S1B) and the proportion of the TIGIT$^+$CD226$^-$ subset was also significantly higher than that of the control. TFH cells in the peripheral blood demonstrated the same trend, but there was no significant difference for the circulating TFR cells (data not shown).
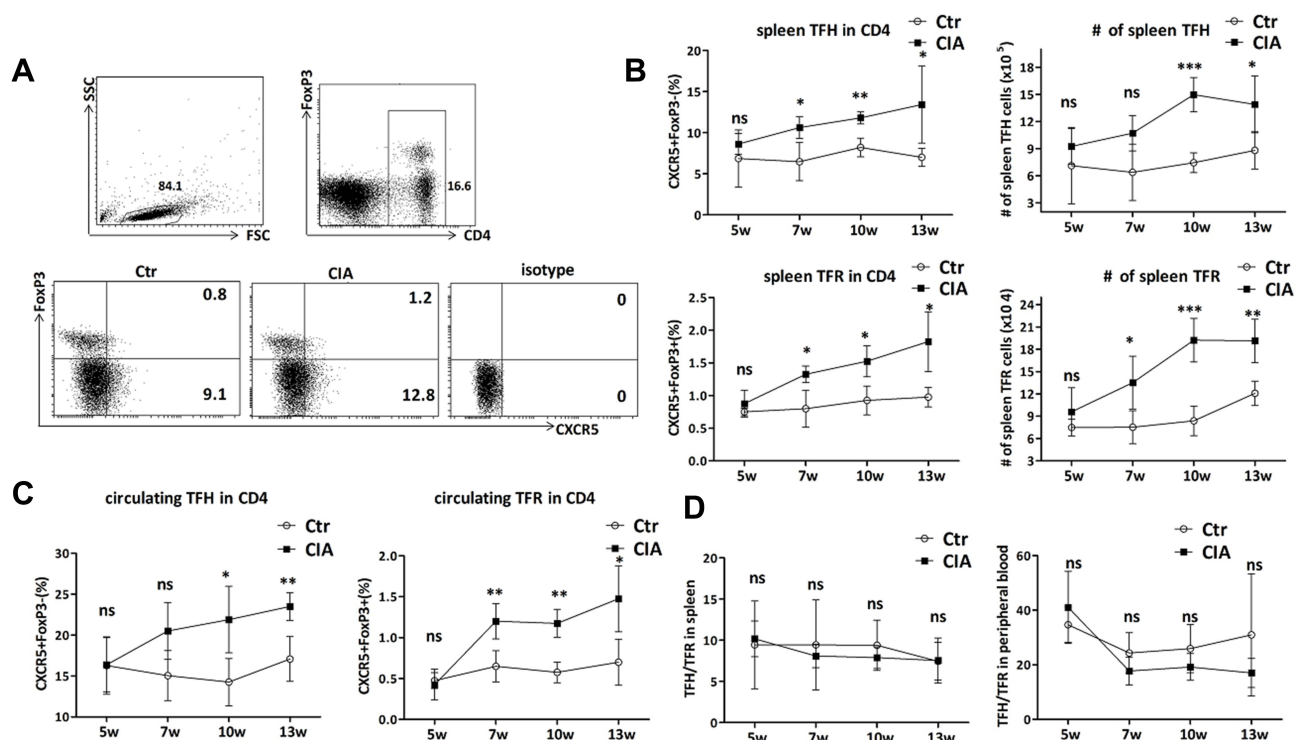
**Figure 2** Analysis of changes in peripheral TFH and TFR cell levels in CIA mice. The CIA mouse model was established in 10-week-old DBA/1 mice. After two immunizations (0w, 3w), the mice were sacrificed at 5w, 7w, 10w, and 13w (n = 4 per timepoint; total = 16 CIA mice). The spleen and PB lymphocytes were separated and analyzed by flow cytometry and compared with that of age-matched Ctr mice (n = 4 per timepoint; total = 16 Ctr mice). (**A**) Representative flow cytometry charts showing the percentages of CD4$^+$CXCR5$^+$FoxP3$^-$ TFH and CD4$^+$CXCR5$^+$FoxP3$^+$ TFR cells in the spleen CD4$^+$ T cells of the two groups at 10w. (**B**) Analysis of the changes in the percentages (left) of TFH and TFR cells (in CD4$^+$ lymphocytes) and their absolute numbers (right) in the spleens of the two groups (n = 4 pairs per timepoint). (**C**) Analysis of the percentages of TFH and TFR cells in circulating CD4$^+$ lymphocytes of the two groups (n = 4 pairs per timepoint). (**D**) TFH/TFR ratios in the spleen (left) and peripheral blood (right) of CIA and Ctr mice (n = 4 pairs per timepoint). Gating was according to isotype controls. Data are the mean ± SD. Results between CIA and Ctr mice for each timepoint were compared using Student's *t* test. ***P < 0.001; **P < 0.01; *P < 0.05; ns, not significant; # total number of indicated cell subsets.
**Abbreviations**: CIA, Collagen-induced arthritis; Ctr, control; PB, peripheral blood; SD, standard deviation; TFH, follicular T helper cell; TFR, follicular regulatory T cell.

TIGIT$^+$ TFH cells have a stronger ability to aid B cells[23] and attenuating CD226 signals increased CXCR5, ICOS, and IL-21 expression by CD155-stimulated germinal center TFH cells.[24] Accordingly, the increase in TIGIT$^+$ and TIGIT$^+$CD226$^-$ TFH cells reflects the enhanced TFH function. Interestingly, the ratios of TIGIT$^+$ and TIGIT+CD226$^-$ TFH cells were both significantly decreased after the antibody treatment (Supplementary Figure S1C).

## PD-1 and ICOS on the Surface of TFH and TFR Cells in CIA Mouse Spleen Were Significantly Increased

Considering that CXCR5 alone may not fully represent mouse peripheral TFH and TFR cells, we studied the changes in PD-1 and ICOS levels on TFH and TFR cells. We measured PD-1$^+$ and/or ICOS$^+$ percentages in CIA mouse TFH and TFR cells over time. The PD-1$^+$, ICOS$^+$, and PD-1$^+$ICOS$^+$ percentages in the CD4$^+$CXCR5$^+$FoxP3$^-$ TFH cells from the CIA mouse spleen were increased significantly after 7w as compared with the control (Figure 4). These molecules can characterize TFH cell function, so the results indirectly suggested that there was relative enhancement of the TFH cell function in the CIA mouse spleen germinal center. In addition, the PD-1$^+$, ICOS$^+$, and PD-1$^+$ICOS$^+$ percentages in CIA mouse spleen TFR cells were also significantly increased (Figure 4).

## Antibody Treatment Normalized Elevated PD-1 and ICOS Expression in CIA Mouse Spleen TFH and TFR Cells

We explored the effect of inflammatory factor antibodies on PD-1 and ICOS expression in CIA mouse TFH and TFR cells. The percentages of PD-1$^+$ and PD-1$^+$ICOS$^+$ cells in the TFH cells decreased significantly after the antibody
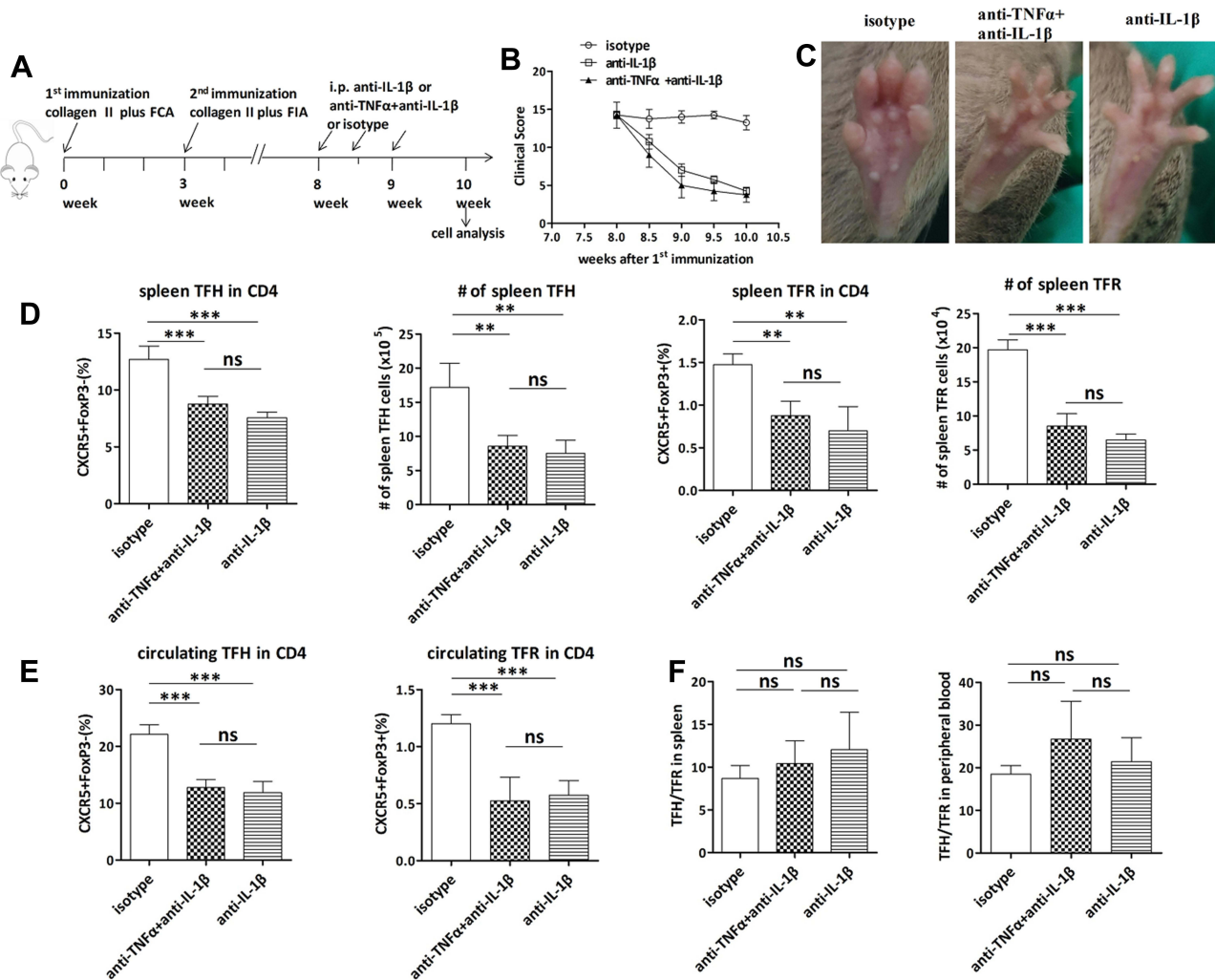
**Figure 3** The effect of antibody treatment on peripheral TFH and TFR cell levels in CIA mice. (**A**) CIA mice were injected with anti-IL-1β or anti-TNFα+anti-IL-1β (n = 4 per treatment option) 1 week before the 10w timepoint with three consecutive injections in a week with a total amount of 0.9 mg. The Ctr mice (n = 4) were injected with isotype IgG. The mice were sacrificed at 10w and the cell subsets were analyzed. (**B** and **C**) The clinical scores of the antibody-treated CIA mice (n = 4 per group) were measured twice each week after injection and compared with the Ctr mice. C. Foot arthritic pictures of the three groups at 10w. (**D**) The treated mice (n = 4) were sacrificed at 10w and the percentages and absolute numbers of CD4$^+$CXCR5$^+$FoxP3$^-$ TFH and CD4$^+$CXCR5$^+$FoxP3$^+$ TFR cells in the spleen CD4 lymphocytes were analyzed and compared with that of the Ctr mice. (**E**) TFH and TFR cell percentages in circulating CD4 lymphocytes were analyzed and compared among the three groups (n = 4 per group). (**F**) Comparison of the TFH/TFR ratios in the spleen (left) and peripheral blood (right) of the three groups (n = 4 per group). Data are from a single experiment representative of two independent experiments. Results were compared using ANOVA and multiple comparisons. Data are the mean with SD. ***P < 0.001; **P < 0.01; *P < 0.05; ns, not significant; # total number of indicated cell subsets.
**Abbreviations**: ANOVA, Analysis of variance; CIA, collagen-induced arthritis; Ctr, control; SD, standard deviation; TFH, follicular T helper cell; TFR, follicular regulatory T cell.

treatment (Supplementary Figure S2). For TFR cells, all three subsets were decreased significantly after the two treatments except that the percentages of ICOS$^+$ cells were not decreased significantly after treatment with anti-IL-1β plus anti-TNFα. These results suggested that PD-1 and ICOS expression on TFH and TFR cells could return to normal after inflammatory factor antibody treatment.

## The Ability of TFH Cells from CIA Mouse Spleen to Secrete IL-21 Was Enhanced and Returned to Normal After Antibody Treatment

To study the ability of TFH and TFR cells to secrete cytokines in CIA mouse germinal centers at the different timepoints, we stimulated lymphocytes with PMA and ionomycin and detected the ability of the TFH and TFR cells to secrete IL-4, IFN-γ, IL-17, IL-21, IL-10, and TNFα. IL-21 secretion by TFH cells was significantly enhanced after the 10w timepoint, which suggests that TFH cell function was enhanced in CIA mouse spleen (Figure 5B). After the 10w timepoint, IL-10
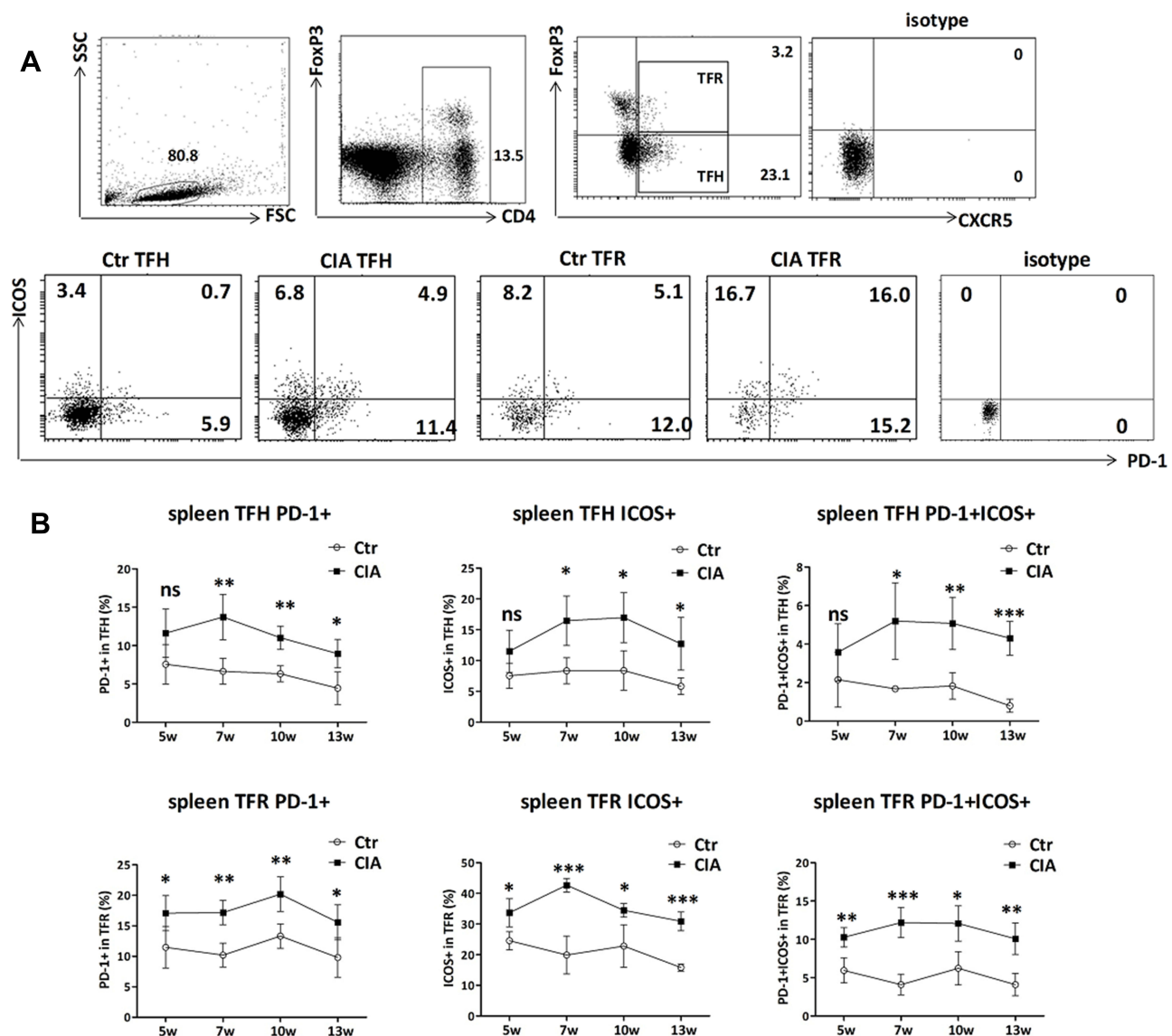
**Figure 4** Analysis of PD-1 and ICOS expression on TFH and TFR cell surface in CIA mouse spleen. The CIA mouse spleen lymphocytes at 5w, 7w, 10w, and 13w (n = 4 per timepoint; total = 16 CIA mice) after the initial immunization were obtained, and PD-1 and ICOS on CD4$^+$CXCR5$^+$FoxP3$^-$ TFH cells and CD4$^+$CXCR5$^+$FoxP3$^+$ TFR cells were detected by flow cytometry and compared with that of age-matched Ctr mice (n = 4 per timepoint; total = 16 Ctr mice). (**A**) Representative flow cytometry plots showing PD-1$^+$ and/or ICOS$^+$ percentages in TFH and TFR cells of CIA and Ctr mice at 10w. The numbers indicate the percentages of the corresponding quadrants in the TFH or TFR cells. (**B**) Comparison of the percentages of PD-1$^+$, ICOS$^+$, and PD-1$^+$ICOS$^+$ subsets in spleen TFH and TFR cells between the CIA and Ctr mice (n = 4 per timepoint). Data are from a single experiment representative of two independent experiments. Data are the mean ± SD. Results between the CIA and Ctr mice for each timepoint were compared using Student's $t$ test. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; ns, not significant.
**Abbreviations**: CIA, Collagen-induced arthritis; Ctr, control; SD, standard deviation; TFH, follicular T helper cell; TFR, follicular regulatory T cell.

secretion by TFR cells from the CIA mice was significantly stronger than that of the control (Figure 5B). The secretion of other cytokines was not significantly different between the CIA and control mice (data not shown). The levels of IL-21 and IL-10 secreted by the TFH and TFR cells, respectively, after treatment were significantly decreased, suggesting that the treatment affected the TFH and TFR cell immune function (Figure 5C).

## TFH and TFR Cell Function in CIA Mouse Spleen Were Enhanced and Returned to Normal After Antibody Treatment

We used an in vitro co-culture system to elucidate the functional changes of TFH and TFR cells in the germinal center of CIA mice. TFH cells from different sources (CIA mice or Ctr mice) were co-cultured with CIA mouse B cells to examine
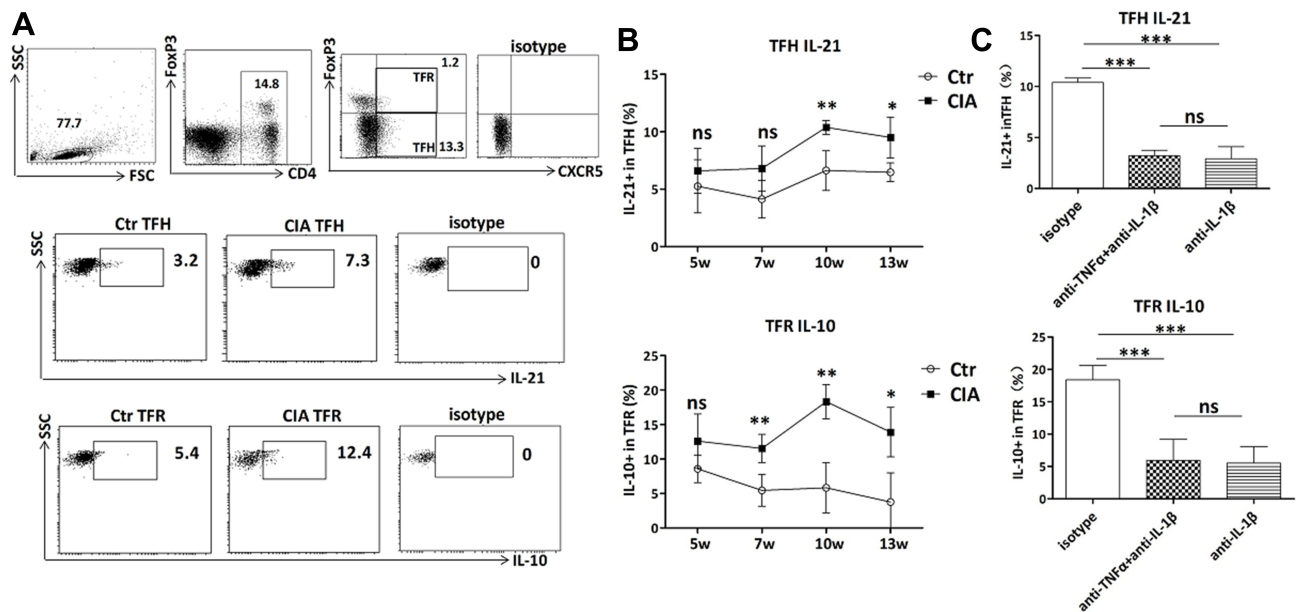
**Figure 5** Analyses of IL-21 and IL-10 secretion capacity of TFH and TFR cells in CIA mouse spleen and their changes upon anti-inflammatory treatment. Spleen lymphocytes from CIA mice and Ctr DBA/1 mice were cultured and stimulated with PMA (50 ng/mL) and ionomycin (1 μg/mL) for 5 h with medium containing brefeldin A. The cells were harvested, stained for surface markers, and intracellularly stained for IL-21 and IL-10. (**A**) Representative flow cytometry dot plots show IL-21$^+$ cells in CD4$^+$CXCR5$^+$FoxP3$^-$ TFH cells and IL-10$^+$ cells in CD4$^+$CXCR5$^+$FoxP3$^+$ TFR cells. (**B**) Comparison of the percentages of IL-21$^+$ TFH cells and IL-10$^+$ TFR cells in the spleens of CIA and Ctr mice at 5w, 7w, 10w, and 13w (n = 4 per timepoint; total = 16 CIA mice) after initial immunization. Results between the CIA and Ctr mice for each timepoint were compared using Student's *t* test. (**C**) CIA mice were injected with anti-IL-1β or anti-TNFα+anti-IL-1β (n = 4 per treatment option) 1 week before the 10w timepoint at three consecutive injections a week with a total amount of 0.9 mg. The Ctr mice (n = 4) were injected with isotype IgG. The mice were sacrificed at 10w and changes in the IL-21$^+$ TFH cells and IL-10$^+$ TFR cells were compared among the groups. Gating was according to isotype controls. Results were compared using ANOVA and multiple comparisons. Data are from a single experiment representative of two independent experiments. Data are the mean with SD. ***P < 0.001; **P < 0.01; *P < 0.05; ns, not significant.
**Abbreviations**: ANOVA, Analysis of variance; CIA, collagen-induced arthritis; Ctr, control; PMA, phorbol 12-myristate 12-acetate; SD, standard deviation; TFH, follicular T helper cell; TFR, follicular regulatory T cell.

TFH cell function. Furthermore, we co-cultured TFR cells from different sources with TFH$^+$ B cells (both from CIA mice) to examine TFR cell function. We measured the functional changes of TFH and TFR cells in regulating antibody secretion by detecting the percentages of GL7$^+$IgG1$^+$ B cells in the culture system and IgG levels in the supernatant. The results indicated that there was relative enhancement of both the function of CIA mouse-derived TFH cells to help B cells and the function of TFR cells for inhibiting TFH cells (Figure 6A-C).

Testing of the TFH and TFR cell functions in the antibody-treated CIA mice revealed a relative reduction in TFH and TFR cell functions after the antibody treatment. The difference between the two treatment options was not significant (Figure 6D and E).

## Discussion

We used a CIA mouse model to study the expression and functional changes of plasmablasts and TFH and TFR subsets in spleen germinal centers and circulating blood. We found increased spleen germinal center IgG1$^+$GL7$^+$ B cells and spleen and peripheral blood plasmablasts. Time-based analysis of the TFH and TFR cells showed that they were increased in late CIA and that their functions were enhanced. The changes in these functional subsets were consistent with the in vitro functional assay results. After treatment with inflammatory factor antibodies, the TFH and TFR subsets and their functions returned to normal. This is the first time an animal model system has been used for studying the function and significance of TFH and TFR cells in the germinal center in the process of RA disease and treatment, which is of notable importance and clinical reference value.

Our research presents two main innovations. First, we used CIA mice to study the changes in splenic TFH and TFR cells, reflecting the dynamic changes of TFH and TFR cell levels in the germinal center in RA, which cannot be achieved in patients with RA. Merely studying the changes in the peripheral blood TFH and TFR cells in patients with RA does
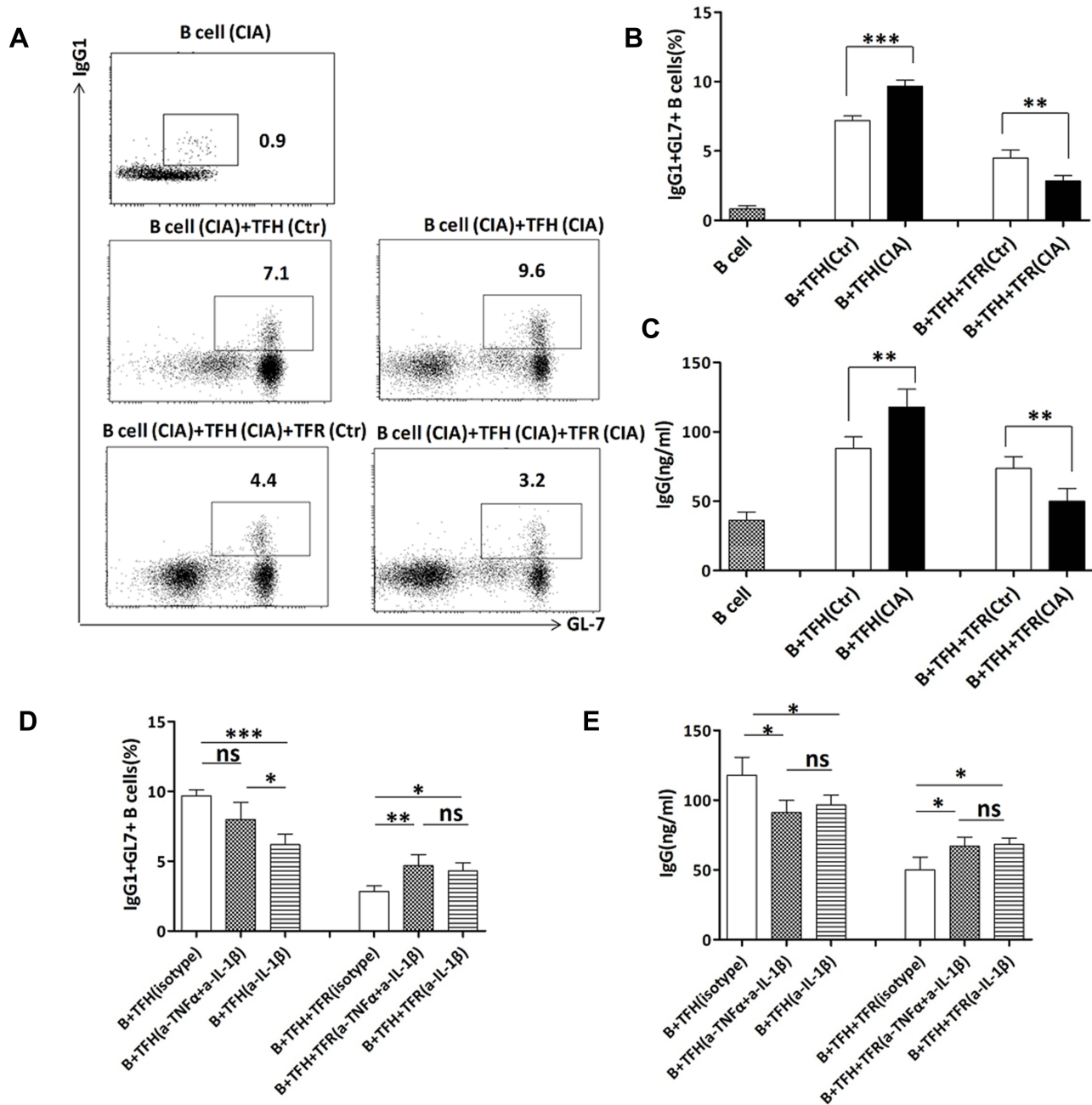
**Figure 6** In vitro culture experiments for detecting the functional changes in TFH and TFR cells in CIA mouse spleen. $CD4^+CXCR5^+CD19^-GITR^-$ TFH cells from 10w CIA mice (n = 4) and Ctr mice (n = 4) were co-cultured with $CD19^+$ B cells (from CIA mouse spleen) for 5 days with anti-CD3 plus anti-IgM. For the TFR cell cultures, $CD4^+CXCR5^+CD19^-GITR^+$ TFR cells from the two groups were added to the wells along with TFH and B cells (both from CIA mouse spleen). (**A**) Representative flow cytometry dot plots of harvested cells showing $GL7^+$ germinal center B cells that secrete IgG1. The numbers indicate the proportions of $GL7^+IgG1^+$ in the $CD19^+$ cells. (**B**) Comparison of the percentages of $GL7^+IgG1^+$ cells in the culture systems. Each group contained cells from the spleens of four mice and each sample was tested once. Results are the mean ± SD. Results between CIA and Ctr mice were compared using Student's *t* test. (**C**) Comparison of IgG concentrations in the supernatant of different cultures. Each group contained cells from four mouse spleens and each sample was tested twice. The data are the mean with SEM. Results between CIA and Ctr mice were compared using Student's *t* test. (**D** and **E**) TFH and TFR cells derived from CIA mice (10w) were injected with anti-inflammatory antibodies (anti-IL-1β or anti-TNFα+anti-IL-1β) or isotype IgG (n = 4 mice per group) and their functions were measured by co-culture. $GL7^+IgG1^+$ cells and IgG levels in the supernatant were measured and compared. The data are the mean with SD. The three groups were compared using ANOVA and multiple comparisons. Data are from a single experiment representative of two independent experiments. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; ns, not significant.
**Abbreviations**: ANOVA, Analysis of variance; CIA, collagen-induced arthritis; PB, peripheral blood; Ctr, control; SD, standard deviation; SEM, standard error of the mean; TFH, follicular T helper cell; TFR, follicular regulatory T cell.

not reflect the full picture of their changes in RA. Second, we studied the effect of inflammatory factor-neutralizing antibody treatment on the TFH and TFR cell changes in vivo and proposed that the antibody therapy affected TFH and TFR cell level and function, thereby affecting the humoral immune response in patients with RA.

TFH and TFR cells play important roles in numerous autoimmune diseases, such as SLE and RA.[20,21,38] Some studies on patients with RA have reported that TFH/TFR ratio is elevated in RA and leads to increased humoral immunity of RA.[39,40] Our study on the peripheral blood of patients with RA determined that circulating TFR/TFH was not upregulated in active RA, but was upregulated in the stable remission stage to suppress humoral immunity.[21] The conclusions on the patients may be related to the differences in patient selection strategies and the differences in the relevant marker molecules used to define TFH and TFR cells. The pre-treatment CIA mice in this research corresponded to the active phase of RA. Here, TFH and TFR cells were upregulated but the TFH/TFR ratio was unchanged, which is consistent with our previous studies on patients with RA.[21]

Although the significance of peripheral TFH and TFR cells in patients with RA has been reported, the changes and significance of these cells in the germinal center of RA remain unclear. In the CIA mouse model, the TFH cells in the spleen germinal center and peripheral blood increased gradually, which coincided with the elevated plasmablast levels in vivo. Our results demonstrated that there was no opposite change between TFR and TFH cells and that TFR cell level and function were upregulated. Although the TFH/TFR ratio did not change significantly, the relative enhancement of B cell function remained as the final result, which indicated that the change in TFH cells played a greater role, which may be related to the relatively higher TFH cell level. At this time, the TFH/TFR ratio may not be the best indicator for reflecting the TFH and TFR cell balance.

In addition, the changes in germinal center TFH and TFR cells have a potential effect on the changes in the peripheral blood TFH and TFR cells. Based on these results and that of our previous studies on patients with RA, the increase in CIA mouse spleen TFH and TFR cells is consistent with the changes in the peripheral blood of patients with active RA.[21] However, TFH and TFR cells were both decreased in the CIA mice after treatment, which is inconsistent with the elevated TFR cells in patients with RA with stable remission.[21] This may be related to the differences in treatment options. The main treatment plan for the patients with RA we studied is methotrexate[21] and its pharmacological mechanism is completely different from that of inflammatory cytokine-neutralizing antibodies. This indicates that different treatment strategies may have different effects on TFH and TFR cell levels in vivo. This difference provides an important reference for different RA treatment options in the future.

In this study, we conducted an in-depth analysis of the phenotypic changes in TFH and TFR cells in CIA mouse spleen. Circulating TFH cells expressing TIGIT exhibited strong B cell help functions.[23] Considering that CD226 can compete with TIGIT[41] and that attenuating CD226 increases CXCR5, ICOS, and IL-21 expression in CD155-stimulated germinal center TFH cells,[24] the downregulation of CD226 relatively enhanced TIGIT function on T cell subsets, so it is speculated that TIGIT$^+$CD226$^-$ TFH cells have a relatively stronger function for helper B cells. Here, both CIA mouse spleen TIGIT$^+$ and TIGIT$^+$CD226$^-$ TFH cells were significantly elevated, which suggests relative enhancement of TFH cell function. ICOS and PD-1 are key molecules expressed on TFH cells, where ICOS is expressed in T cells after activation.[42,43] ICOS is essential for TFH cell development and plays important roles in B cell maturation and activation by combining with ICOS-L expressed on B cells.[44] Circulating ICOS$^+$PD-1$^+$ TFH cells play important roles in several diseases.[45,46] The PD-1$^+$, ICOS$^+$, and PD-1$^+$ICOS$^+$ percentages in CD4$^+$CXCR5$^+$FoxP3$^-$ TFH cells in CIA mouse spleen were increased significantly, suggesting that TFH cell function was enhanced, which was also proven by the enhanced IL-21 secretion and the in vitro function assays. The PD-1–PD-L1 pathway plays a critical role in TFR cell generation and function and PD-1–PD-L1 signaling inhibited TFR cell differentiation and controlled their suppressive ability.[16] In our research, PD-1$^+$ TFR cells were increased in the CIA mouse spleens, but an in vitro functional assay demonstrated relative enhancement of TFR cell function. This inconsistency may be because although PD-1 expression on TFR cells can reflect the activation level of the signaling pathway to a certain extent, the two may not be completely consistent. We also found that ICOS expression is upregulated in the CIA mouse spleen TFR cells, but the detailed function of ICOS in TFR expression remains unclear and requires elaboration in future studies.

IL-21 plays a role in TFH cell formation and B cell growth and survival.[47–49] There was relative enhancement of the ability of TFH cells to secrete IL-21 in CIA mouse spleen, which suggests that TFH cells have enhanced functions in the

process of CIA. IL-21 upregulation may promote TFH cell function in CIA. IL-10-producing TFR cells played a major role in maintaining the germinal center reaction and therefore acted as helper cells.[50,51] There was relatively enhanced ability of TFR cells to secrete IL-10, but functional studies determined that the ability of TFR cells to inhibit TFH cells was enhanced, which manifested as a decrease in the level of antibodies secreted by B cells. This contradiction can be explained by the fact that TFR cells not only function through IL-10, but also directly function on TFH cells. Although the level of IL-10+ TFR cells was increased, the final result was an increase in TFR inhibition function.

This research also focused on the impact of inflammatory factor-neutralizing antibody therapy on TFH and TFR cells. Based on previous reports, we selected two treatment options: anti-TNFα+anti-IL-1β and anti-IL-1β alone.[52,53] The results showed that the TFH and TFR cell levels, subpopulations, and functions returned to normal after the antibody treatment. This result indicates that inflammatory factors play an important role in the increase and functional enhancement of TFH and TFR cells in CIA mice. The results demonstrated that the difference between the two treatment options was not obvious, which suggests that the IL-1β antibody played a relatively greater role in the treatment. Very complex work is needed to elucidate why the use of inflammatory factor-neutralizing antibodies can significantly restore the upregulated levels and functions of TFH and TFR cells in CIA mice. The first possible reason is that they significantly changed the in vivo environment of the inflammatory factors, and the changes of these inflammatory factors caused further changes in the cytokine network, which ultimately affected TFH and TFR cell differentiation and function. In addition, inflammatory cytokine antibodies can significantly act on cells with the corresponding receptors, which may include T cells themselves, and B cells and antigen-presenting cells that interact with T cells. The interaction of these cells with antibodies leads to TFH and TFR cell changes and further affects their level and function.

Our study has many shortcomings. One important point is that we focused on the changes in T cell subpopulations but did not pay more attention to the dynamic changes in B cells and antibody secretion in the CIA mice. Nevertheless, we quantitatively measured B cells through in vitro culture function assays to compensate. The selection of the age-matched control mice may be a limitation, but the mice that underwent the other treatment methods were also less representative of the situation for the control individuals, ie, only FCA was injected. Therefore, age-matched healthy mice can only be used as the control group. Nonetheless, the question remains as to whether the TFH and TFR cell changes are factors that promote RA onset or are merely passive changes in RA onset. Our experimental results cannot answer this question, which requires clarification in the future. Although the CIA model is a classic RA animal model, it needs further verification in the patient population, especially the changes in TFH and TFR cells in patients with RA before and after inflammatory factor-neutralizing antibody treatment.

## Conclusions

In this study, TFH and TFR cells in spleen germinal centers were increased in RA and their functions were enhanced. Furthermore, the TFH and TFR subsets and their functions returned to normal after the anti-inflammatory antibody treatment. The changes in the TFH and TFR cell subsets and functions in the spleen germinal center in RA contributed to comprehensive understanding of the changes of humoral immune-related cell subsets during RA. These findings provide new insight into the changes in immune balance in RA pathogenesis. TFH and TFR cell changes after anti-inflammatory antibody treatment of RA also demonstrate important clinical significance.

## Abbreviations

ANOVA, Analysis of variance; ARRIVE, Animal Research: Reporting of In Vivo Experiments guidelines; Bcl-6, B cell lymphoma 6; Blimp1, B lymphocyte-induced maturation protein-1; CIA, collagen-induced arthritis; CII, type II collagen; FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant; FoxP3, forkhead box P3; GITR, glucocorticoid-induced TNF receptor; ICOS, inducible costimulator; IFN, interferon; IL, interleukin; PD-1, programmed cell death 1; PD-L1, programmed death ligand 1; PMA, phorbol 12-myristate 12-acetate; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; TFH, follicular helper T cells; TFR, follicular regulatory T cells; TIGIT, T cell Ig and ITIM domain; TNF, tumor necrosis factor; Treg, regulatory T cells.

## Data Sharing Statement

All supporting data in this study are available from the corresponding author on reasonable request.

## Ethics Approval Statement for Animal Studies

All mice received humane care and the research protocol was approved by the ethical board of Peking University People's Hospital.

## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

## Disclosure

The authors report no conflicts of interest in this work.

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