

# High frequency of circulating follicular helper T cells is correlated with B cell subtypes in patients with ankylosing spondylitis

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Received October 6, 2017; Accepted February 2, 2018

DOI: 10.3892/etm.2018.5991

**Abstract.** T follicular helper (Tfh) cells are known to support effector B cells and enhance autoimmunity; however, the association between the Tfh cells and B cells in ankylosing spondylitis (AS) is unclear. The aim of the present study was to measure the frequency of circulating cluster of differentiation (CD)4<sup>+</sup> C-X-C chemokine receptor type 5 (CXCR5)<sup>+</sup> Tfh cells and B cell subtypes in peripheral blood from patients with AS, and evaluate the correlation of these factors. Percentages of peripheral blood circulating CD4<sup>+</sup>CXCR5<sup>+</sup> Tfh cells and B cell subtypes were measured via flow cytometry and the disease activity of individual patients was measured using the Bath AS Disease Activity Index (BASDAI). The potential association among these measures was analyzed via Spearman's or Pearson's correlations. In comparison with those in healthy controls (HC), significantly increased percentages of CD4<sup>+</sup>CXCR5<sup>+</sup> cTfh, CD4<sup>+</sup>CXCR5<sup>+</sup> programmed death 1<sup>+</sup>, CD4<sup>+</sup>CXCR5<sup>+</sup> inducible T cell costimulator (ICOS)<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>CXCR5<sup>+</sup>interleukin (IL)-21<sup>+</sup> T cells, CD19<sup>+</sup>CD27<sup>high</sup> plasmablast and CD19<sup>+</sup>CD38<sup>+</sup> antibody-secreting B cells were detected in patients with AS, whereas there was no significant difference in CD19<sup>+</sup>CD27<sup>+</sup> naïve B cells and CD19<sup>+</sup>CD27<sup>+</sup> memory B cells. When Patients with AS were divided into high and low activity groups, significantly higher percentages of CD4<sup>+</sup>CXCR5<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>CXCR5<sup>+</sup>IL-21<sup>+</sup> T cells, CD19<sup>+</sup>CD27<sup>+</sup> naïve B cells and CD19<sup>+</sup>CD38<sup>+</sup> antibody-secreting B cells, and lower CD19<sup>+</sup>CD27<sup>+</sup> memory B cells were detected in high activity AS group compared with the low activity AS group. In addition, percentages of CD4<sup>+</sup>CXCR5<sup>+</sup> circulating (c)Tfh, CD3<sup>+</sup>CD8<sup>+</sup>CXCR5<sup>+</sup>IL-21<sup>+</sup> T and CD19<sup>+</sup>CD38<sup>+</sup> antibody-secreting B cells were positively

correlated with BASDAI values. Furthermore, the percentage of CD4<sup>+</sup>CXCR5<sup>+</sup> cTfh cells was positively correlated with CD19<sup>+</sup>CD38<sup>+</sup> antibody-secreting B cells and the percentage of CD3<sup>+</sup>CD8<sup>+</sup>CXCR5<sup>+</sup>IL-21<sup>+</sup> T cells was positively correlated with CD19<sup>+</sup>CD27<sup>+</sup> naïve B cells in patients with AS. These findings suggest that CD4<sup>+</sup>CXCR5<sup>+</sup> cTfh, CD3<sup>+</sup>CD8<sup>+</sup>CXCR5<sup>+</sup>IL-21<sup>+</sup> T and CD19<sup>+</sup>CD38<sup>+</sup> antibody-secreting B cells may participate in the pathogenesis of AS because of their distinct functions. As such, levels of cTfh and B cell subtypes may be a useful biomarker for the evaluation of disease activity in patients with AS.

## Introduction

Ankylosing spondylitis (AS) is a chronic, progressive autoimmune disease that, theoretically, affects the axial and sacroiliac joints. More importantly, the majority of patients with AS eventually develop spine malformations, leading to functional incapacity (1). Although, the pathogenesis of AS remains unknown, an increasing amount of research has been performed to elucidate it.

Previous studies have reported elevated percentages of peripheral blood T helper (Th)1, Th17 and Th22 cells, and decreased percentages of regulatory T cells in patients with AS (2-4). However, to the best of our knowledge, there have been only two studies regarding a novel, distinct subgroup of cluster of differentiation (CD)4<sup>+</sup> T cells, known as follicular helper T cells (Tfh), in patients with AS (5,6). A number of previous studies have demonstrated that aberrant expression of circulating (c)Tfhs may mediate the development of autoimmune diseases, such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), multiple sclerosis and Sjogren's syndrome (7-10). Tfhs are known as specialized providers of help to B cells and persistently express C-X-C chemokine receptor type 5 (CXCR5), which drives Tfh migration into B cell follicles in response to the specific ligand CXCL13, which is expressed by follicular dendritic cells (11). Morita *et al* (12) have identified CD4<sup>+</sup>CXCR5<sup>+</sup> T cells as cTfhs as they share similar functional properties with Tfh cells. Furthermore, Tfh cells express programmed death 1 (PD-1), inducible T cell costimulator (ICOS), CD40 ligand (CD40L) and interleukin (IL)-21, which not only serve as excellent markers for the identification of Tfh cells, but can also

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**Key words:** ankylosing spondylitis, B cell subtypes, circulating follicular helper T cells, C-X-C chemokine receptor type 5, interleukin-21

interact with B cell surface ligands to promote the formation of germinal centers (GC), the differentiation of B cells and antibody production (13,14).

In addition, other previous studies have reported increased percentages of B cells and high levels of autoantibodies in patients with AS (15,16). However, very few studies have focused on the phenotypic and functional status of B cells in different disease activities of AS. Several reports have demonstrated that the abnormal distribution of B cell subtypes may participate in the pathogenesis of autoimmune diseases, such as RA, SLE and IgA nephropathy (17-19). A previous study of primary Sjogren's syndrome (8) has reported that abnormal increases of CD4<sup>+</sup>CXCR5<sup>+</sup> Tfh cells and B cell subsets in the salivary gland were significantly correlated with serum anti-nuclear antibody titers. A further study (20) revealed higher percentages of activated B and Tfh cells in patients with RA as well as regulation of B cell activation via active Tfh cells in the process of RA.

As such, the aim of the present study was to investigate changes in the distribution of B cell subtypes and whether Tfh is associated with the distribution of B cell subtypes in patients with AS. The frequency of cTfhs and different stages of differentiated B cells were investigated in 65 patients with AS as well as in 20 gender and age-matched healthy participants. The present findings suggest that certain subtypes of cTfh cells and B cells may participate in the pathogenesis of AS due to their distinct functions, and the percentages of cTfhs and B cell subtypes may be useful as a valuable measure for evaluating disease activity in patients with AS.

## Materials and methods

**Patients and controls.** A total of 65 patients with AS were recruited sequentially at the outpatient clinic of Guizhou Medical University Hospital (Guiyang, China) from September 2014 to October 2015. All patients fulfilled the 1984 modified New York criteria (21), which is the criterion for diagnosing AS. A further 20 healthy age- and sex-matched individuals with no history of inflammatory or autoimmune diseases were recruited during the same period as healthy controls (HC). Patients with AS were excluded if they had any other chronic inflammatory and autoimmune disorders such as diabetes, multiple sclerosis or inflammatory bowel disease, or if they were currently receiving treatment with non-steroidal anti-inflammatory drugs, steroids, or other immunosuppressants. The disease activity of individual patients was measured using the Bath AS Disease Activity Index (BASDAI) (22). The scores for each criterion ranged from 0-10, with high activity defined as a BASDAI score  $\geq 4$  and low activity defined as a BASDAI score  $< 4$ . All patients provided written informed consent prior to their inclusion in the present study and the experimental protocol was approved by the institutional ethics committee of Guizhou Medical University Hospital. The characteristics of the study subjects are summarized in Table I.

**Flow cytometry analysis.** Peripheral venous blood samples were collected following overnight fasting. Expression of human leukocyte antigen (HLA)-B27 in T cells of patients with AS was characterized via flow cytometry analysis. Fresh peripheral blood was incubated with mixed

fluorescein-labeled antibodies [4.2  $\mu\text{g/ml}$  fluorescein isothiocyanate (FITC)-anti-CD3 and 5.0  $\mu\text{g/ml}$  phycoerythrin (PE)-anti-HLA-B27; cat. no. 340183; BD Biosciences, Franklin Lakes, NJ, USA] at room temperature for 15 min. Following lysis of erythrocytes with hemolysin (cat. no. 349202; BD Biosciences) at room temperature for 8 min and washing with PBS, white blood cells were subjected to BD FACS Canto II flow cytometry analysis using FACSCanto Clinical software version 2.4 (BD Biosciences).

Peripheral venous blood in duplicate was incubated with 12  $\mu\text{g/ml}$  allophycocyanin (APC)-cyanine (CY)7-anti-CD4 (cat. no. 341115; BD Biosciences), peridinin-chlorophyll-protein complex (PERCP)-CY5.5-anti-CXCR5 (cat. no. 562781; BD Pharmingen, San Diego, CA, USA), 0.5 mg/ml FITC-anti-PD-1 (FITC-anti-CD279; cat. no. 561035; BD Pharmingen; BD Biosciences) and 0.2 mg/ml PE-anti-ICOS (PE-anti-CD278; cat. no. 552146; BD Pharmingen; BD Biosciences) antibodies in the dark at room temperature for 15 min. Control cells were also from patients with AS and were incubated with 12  $\mu\text{g/ml}$  APC-CY7-anti-CD4, 25  $\mu\text{g/ml}$  PERCP-CY5.5-anti-immunoglobulin G (IgG)1 (cat. no. 347202; BD Biosciences), 50  $\mu\text{g/ml}$  FITC-anti-IgG (cat. no. 349041; BD Biosciences) and 50  $\mu\text{g/ml}$  PE-anti-IgG1 (cat. no. 349043; BD Bioscience) in the dark at room temperature for 15 min, which is a negative control of flow analysis. Controls were isotyped, which helped eliminate non-specific fluorescent interference in flow analysis. In addition, peripheral blood in duplicate was stained with 0.05 mg/ml APC-anti-CD19 (cat. no. 340437; BD Biosciences), 8  $\mu\text{g/ml}$  FITC-anti-CD27 (cat. no. 340424; BD Biosciences), 25  $\mu\text{g/ml}$  PE-CY7-anti-CD38 (cat. no. 335790; BD Biosciences) or isotype-matched controls (BD Bioscience) at room temperature for 15 min. Following lysis of erythrocytes with hemolysin and washing with PBS, white blood cells were subjected to FACS Canto II flow cytometry analysis using a FACSDiva software version 6.1.3 (BD Biosciences).

**Analysis of cytokine production.** Peripheral blood in duplicate was stimulated with 50 ng/ml phorbol myristate acetate (PMA; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and 1  $\mu\text{g/ml}$  ionomycin (Sigma-Aldrich; Merck KGaA) with 10% fetal calf serum (Zhejiang Tianhang Biotechnology Co., Ltd., Zhejiang, China) in RPMI 1640 medium (Boster Biological Technology, Pleasanton, CA, USA) at 37°C in an incubator with 5% CO<sub>2</sub> for 1.5 h and subsequently cultured for a further 4.5 h in the presence of GlogiStop (cat. no. 554724; BD Biosciences) at 37°C. As the expression of CD4 epitope would be decreased following the stimulation of PMA and ionomycin (23), CD3<sup>+</sup>CD8<sup>+</sup> T was used to represent CD3<sup>+</sup>CD4<sup>+</sup> T cells. The stimulated cells were incubated with 100  $\mu\text{g/ml}$  FITC-anti-CD3 (cat. no. 349201; BD Biosciences), 50  $\mu\text{g/ml}$  PE-CY7-anti-CD8 (cat. no. 335822; BD Biosciences) and 3  $\mu\text{g/ml}$  PE-anti-CD69 (cat. no. 341652; BD Pharmingen; BD Biosciences) antibodies in the dark at room temperature for 15 min. Control cells were incubated with 100  $\mu\text{g/ml}$  FITC-anti-CD3, 50  $\mu\text{g/ml}$  PE-CY7-anti-CD8 and 50  $\mu\text{g/ml}$  PE-anti-IgG1 (cat. no. 349043; BD Biosciences) antibodies in the dark at room temperature for 15 min. Following lysis of erythrocytes and washing with PBS, the expression of CD69 in CD3<sup>+</sup>CD8<sup>+</sup> (CD3<sup>+</sup>CD4<sup>+</sup>) T lymphocytes was analyzed using the BD FACS Canto II flow cytometer. When the proportion

Table I. Characteristics of patients with AS and HC.

Characteristic	AS patients (n=65)	HC (n=20)
Age, years (mean $\pm$ SD)	27.8 $\pm$ 8.5	31.4 $\pm$ 7.4
Sex (male/female)	55/10	15/5
HLA-B27 positive, n	64	0
BASDAI score (mean $\pm$ SD)	4.38 $\pm$ 1.73	-

AS, ankylosing spondylitis; HC, healthy control; BASDAI, Bath AS Disease Activity Index; SD, standard deviation.

of CD3<sup>+</sup>CD8<sup>+</sup>CD69<sup>+</sup> T cells in CD3<sup>+</sup>CD8<sup>+</sup> T lymphocytes was >90%, the subsequent experiments were performed.

The stimulated cells (200  $\mu$ l) in duplicate were incubated with 100  $\mu$ g/ml FITC-anti-CD3, 50  $\mu$ g/ml PE-CY7-anti-CD8 and PERCP-CY5.5-anti-CXCR5 antibodies at room temperature for 15 min. The cells were subsequently fixed with 50  $\mu$ l BD FACS permeabilizing solution A (cat. no. 347692; BD Biosciences) at room temperature for 5 min according to the manufacturer's protocol, and then permeabilized with BD FACS permeabilizing solution B (cat. no. 347692; BD Biosciences) and incubated with PE-anti-IL-21 antibody (cat. no. 562042; BD Pharmingen; BD Biosciences) or PE-anti-IgG1 antibody at room temperature for 20 min, followed by BD FACS Canto II flow cytometry analysis of IL-21 expression.

**Statistical analysis.** Statistic analyses were performed using SPSS (version 20.0; IBM Corp., Armonk, NY, USA). Normally distributed data are expressed as the mean  $\pm$  standard deviation, whereas skewed data are presented as the median. Kolmogorov-Smirnov test was used to assess the distribution of the data. In addition, Levene's test was used to evaluate the homogeneity of variances. The significance of the difference between multiple groups was performed using one-way analysis of variance with a post-hoc Student-Newman-Keuls test and the significance of difference between two groups was evaluated with two-tailed Student's t-test. Spearman correlation coefficient or Pearson correlation coefficient with two-tailed P-values were determined in the analysis of correlations. Two-sided P<0.05 was considered to indicate a statistically significant difference.

## Results

**Increased percentages of CD4<sup>+</sup>CXCR5<sup>+</sup> cTfh cells in patients with AS.** To investigate the potential role of Tfh cells and their surface molecules in the development of AS, the frequency of peripheral blood CD4<sup>+</sup> T and CD4<sup>+</sup>CXCR5<sup>+</sup> cTfh cells were characterized with flow cytometry analysis (Fig. 1A). It was demonstrated that there was a decreased frequency of CD4<sup>+</sup> T cells in patients with AS compared with HC (P<0.01; Fig. 1B). conversely, it was demonstrated that the percentages of CD4<sup>+</sup>CXCR5<sup>+</sup> cTfh cells, CD4<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>+</sup> and CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>+</sup> T cells were significantly increased in patients with AS compared with HC (P<0.01 for all; Fig. 1C-E). Furthermore, the percentage of CD4<sup>+</sup>CXCR5<sup>+</sup>

cTfh was significantly elevated in the high activity AS group (BASDAI score  $\geq$ 4; n=41) compared with that in the low activity AS group (BASDAI score <4; n=24; P=0.001) and in HC (n=20; P<0.001; Fig. 1C). However, there was no significant difference observed in the frequency of peripheral blood CD4<sup>+</sup> T cells, CD4<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>+</sup> and CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>+</sup> T cells between the high and low activity AS groups (Fig. 1B, D and E).

**Increased percentage of CD3<sup>+</sup>CD8<sup>+</sup>CXCR5<sup>+</sup>IL-21<sup>+</sup> T cells in patients with AS.** To determine whether the lymphocytes were activated, the expression of CD69 in stimulated T lymphocytes was measured using flow cytometry, as presented in Fig. 2A. As such, these experiments were performed only when the percentage of the CD3<sup>+</sup>CD8<sup>+</sup>CD69<sup>+</sup> T cells in CD3<sup>+</sup>CD8<sup>+</sup> T cells was  $\geq$ 90%.

The intracellular production of IL-21 by CD3<sup>+</sup>CD8<sup>+</sup>(CD3<sup>+</sup>CD4<sup>+</sup>) T cells was initially assessed using flow cytometry (Fig. 2A), which revealed that CD3<sup>+</sup>CD8<sup>+</sup> T cells producing IL-21 were significantly increased in peripheral blood of patients with AS compared with that in HC (P<0.001; Fig. 2B). As IL-21 is secreted by CD4<sup>+</sup> T cells, including Tfh cells, Th17 cells and natural killer T cells (24), CXCR5 expression was used to dichotomize CD3<sup>+</sup>CD8<sup>+</sup>IL-21<sup>+</sup> T cells into CD3<sup>+</sup>CD8<sup>+</sup>CXCR5<sup>+</sup>IL-21<sup>+</sup> T and CD3<sup>+</sup>CD8<sup>+</sup>CXCR5<sup>-</sup>IL-21<sup>+</sup> T portions (Fig. 2A). Compared with HC, the percentages of CD3<sup>+</sup>CD8<sup>+</sup>CXCR5<sup>+</sup>IL-21<sup>+</sup> T and CD3<sup>+</sup>CD8<sup>+</sup>CXCR5<sup>-</sup>IL-21<sup>+</sup> T cells were significantly increased in patients with AS (P<0.001 for all; Fig. 2C and D). The percentage of CD3<sup>+</sup>CD8<sup>+</sup>CXCR5<sup>+</sup>IL-21<sup>+</sup> T cells were significantly increased in the high activity AS group (n=33; some patients with AS failed the test and were not included in statistical analysis) compared with that in the low activity AS group (n=21) and in HC (P<0.001 for all; Fig. 2C), and a similar increase was also observed in the percentages of CD3<sup>+</sup>CD8<sup>+</sup>IL-21<sup>+</sup> T and CD3<sup>+</sup>CD8<sup>+</sup>CXCR5<sup>-</sup>IL-21<sup>+</sup> T cells (P<0.001 for all; Fig. 2B and D). This suggests that different cellular sources of IL-21 are associated with the development of AS in this cellular population.

**Aberrant distribution of peripheral blood B cell subtypes in patients with AS.** As presented in Fig. 3, the distribution of B cell subtypes was also measured using flow cytometry. The percentages of CD19<sup>+</sup>CD27<sup>high</sup> plasmablasts and CD19<sup>+</sup>CD38<sup>+</sup> antibody-secreting B cells in patients with AS were significantly higher than those in HC (P<0.001 for all; Fig. 3C and F). There was no significant difference in the frequency of CD19<sup>+</sup> B cells, CD19<sup>+</sup>CD27<sup>-</sup> naïve B cells and CD19<sup>+</sup>CD27<sup>+</sup> memory B cells between patients with AS and HC (Fig. 3B, D and E). The percentages of CD19<sup>+</sup>CD38<sup>+</sup> antibody-secreting B cells and CD19<sup>+</sup>CD27<sup>-</sup> naïve B cells were also significantly increased in the high activity AS group (n=24) compared with the low activity AS group (n=22) and HC groups (P<0.05; Fig. 3C and D). In contrast, the frequency of CD19<sup>+</sup>CD27<sup>+</sup> memory B cells was significantly decreased in the high activity patients with AS, compared with the low activity AS group (P<0.05; Fig. 3E). Notably, the percentage of CD19<sup>+</sup>CD38<sup>+</sup> antibody-secreting B cells was positively correlated with the BASDAI values in patients with AS (r=0.329, P=0.007; Fig. 3G). However, there was no significant correlation between the BASDAI values and

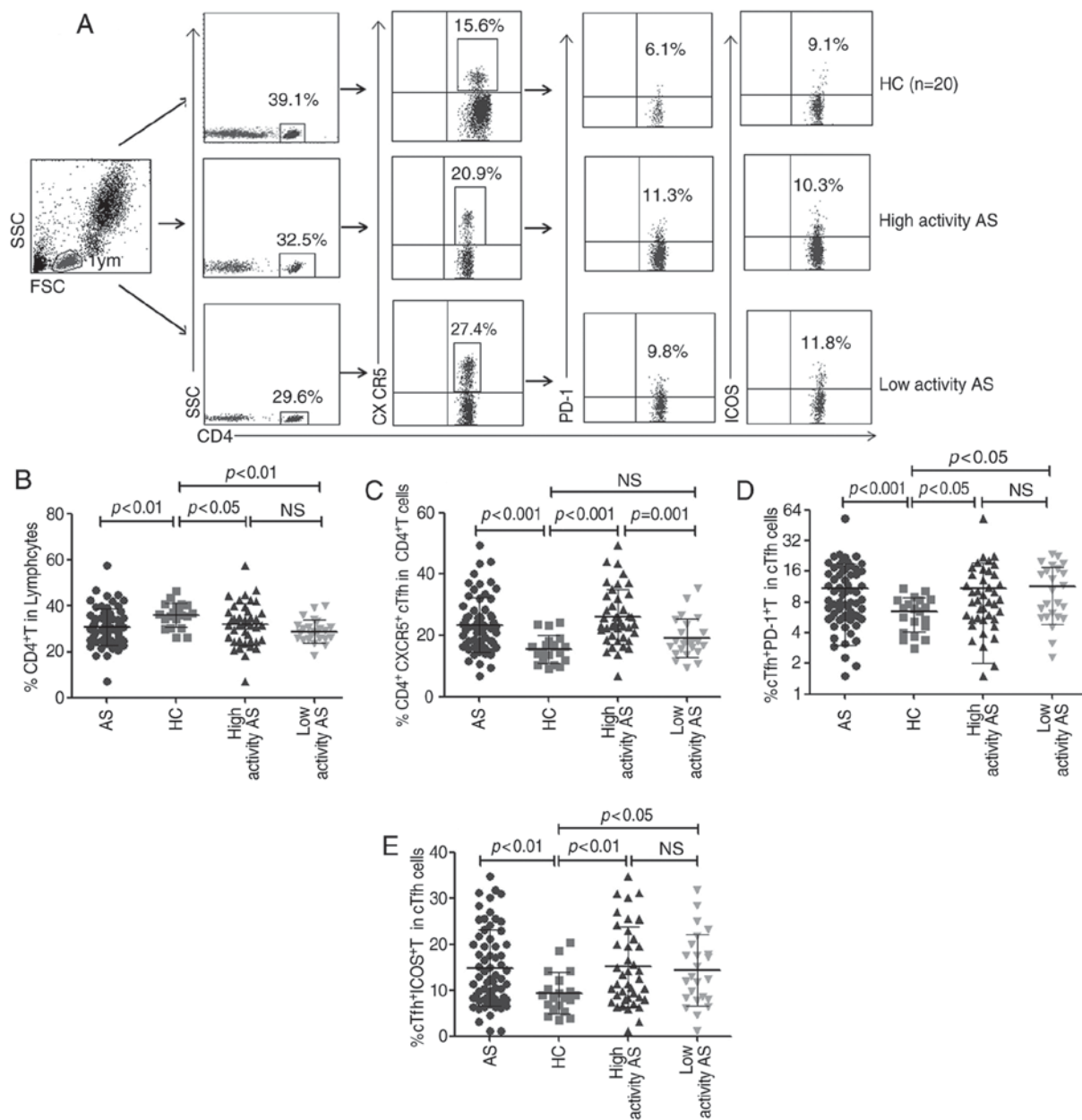


Figure 1. Flow cytometry analysis of the frequency of peripheral blood cTfh cells. Fresh peripheral blood was stained with anti-CD4, anti-CXCR5, anti-PD-1 and anti-ICOS antibodies. Following gating CD4<sup>+</sup> T cells, frequencies of CD4<sup>+</sup>CXCR5<sup>+</sup> cTfh cells, CD4<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>+</sup> T cells and CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>+</sup> T cells were analyzed. (A) Representative images of flow cytometry analysis. Percentages of (B) CD4<sup>+</sup> T, (C) CD4<sup>+</sup>CXCR5<sup>+</sup> cTfh, (D) CD4<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>+</sup> T and (E) CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>+</sup> T cells were compared between patients with AS (n=65) and HC (n=20). cTfh, circulating follicular helper T cells; CD, cluster of differentiation; CXCR5, C-X-C chemokine receptor type 5; PD-1, programmed death 1; ICOS, inducible T cell costimulator; NS, no significant differences; HC, healthy controls; AS, ankylosing spondylitis; SSC, side scatter data; FSC, forward scatter data.

the frequency of other B cell subtypes in patients with AS (data not shown).

*Increase in peripheral blood cTfh cells was positively correlated with disease activities in patients with AS.* The percentage of CD4<sup>+</sup>CXCR5<sup>+</sup> cTfh and CD3<sup>+</sup>CD8<sup>+</sup>CXCR5<sup>+</sup>IL-21<sup>+</sup> T cells was positively correlated with the values of BASDAI ( $r=0.334$ ,  $P=0.006$ ;  $r=0.594$ ,  $P<0.001$ ; Fig. 4A and B). A similar correlation was observed in the percentages of CD3<sup>+</sup>CD8<sup>+</sup>IL-21<sup>+</sup> T and CD3<sup>+</sup>CD8<sup>+</sup>CXCR5<sup>+</sup>IL-21<sup>+</sup> T cells with the BASDAI values ( $r=0.558$ ,  $P<0.001$ ;  $r=0.561$ ,  $P<0.001$ ; Fig. 4C and D). However, there was no significant correlation between percentages of other cTfh cell phenotypes

(CD4<sup>+</sup>CXCR5<sup>+</sup>PD-1 and CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>+</sup> T cells) and the BASDAI values in patients with AS (data not shown).

*Association between cTfh cells and B cells in patients with AS.* The percentage of CD4<sup>+</sup>CXCR5<sup>+</sup> cTfh cells was positively correlated with the frequency of CD19<sup>+</sup>CD38<sup>+</sup> antibody-secreting B cells in patients with AS ( $r=0.38$ ,  $P=0.002$ ; Fig. 5A). Similarly, the percentage of CD3<sup>+</sup>CD8<sup>+</sup>CXCR5<sup>+</sup>IL-21<sup>+</sup> T cells was also positively correlated with the frequency of CD19<sup>+</sup>CD27<sup>+</sup> naïve B cells ( $r=0.321$ ,  $P=0.034$ ; Fig. 5B). However, there was no significant association between the percentages of other phenotypes of cTfh cells and the subtypes of B cells evaluated in these patients (data not shown). These

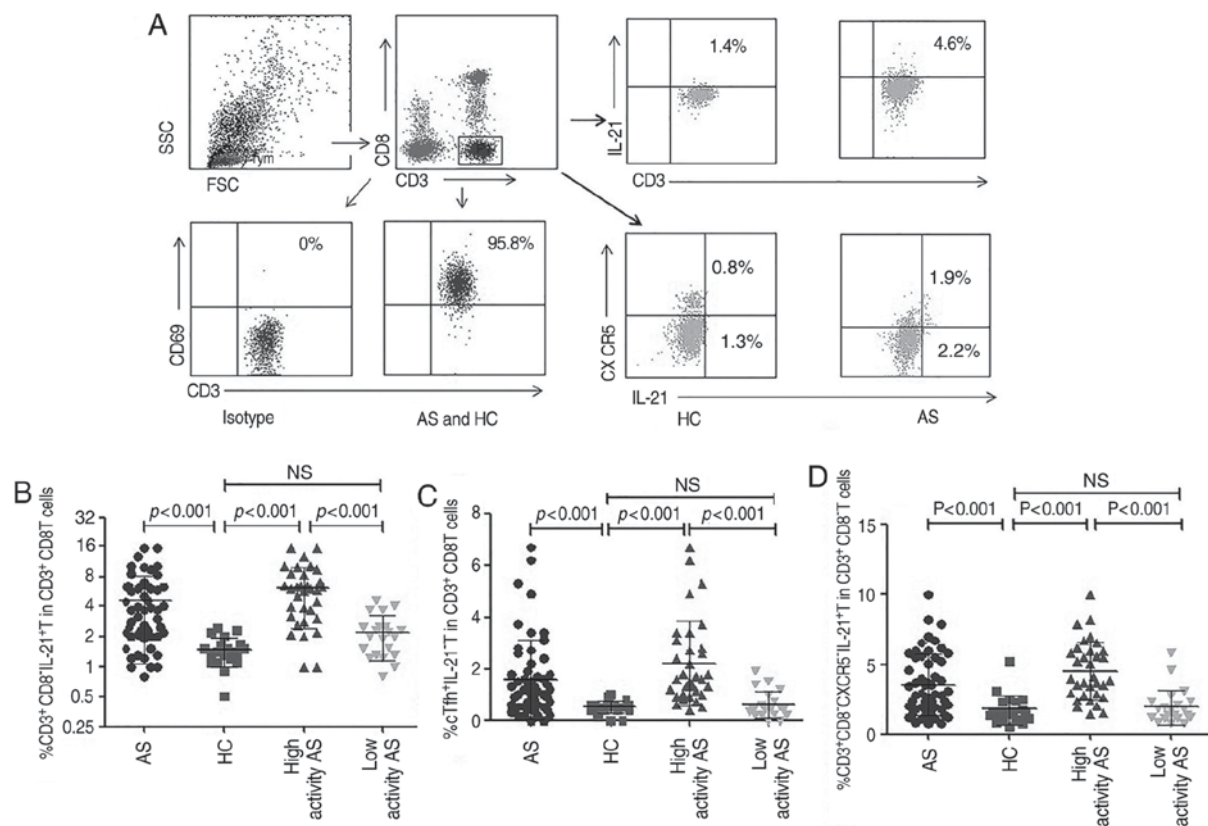


Figure 2. Flow cytometry analysis of different cellular sources of IL-21. The cells were characterized using flow cytometry by gating living lymphocyte cells and then CD3<sup>+</sup>CD8<sup>+</sup> T cells. The frequencies of CD3<sup>+</sup>CD8<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>CXCR5<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>CXCR5<sup>+</sup> T cells producing IL-21 were then analyzed. (A) Representative flow cytometry analysis. Percentages of (B) CD3<sup>+</sup>CD8<sup>+</sup>, (C) CD3<sup>+</sup>CD8<sup>+</sup>CXCR5<sup>+</sup> and (D) CD3<sup>+</sup>CD8<sup>+</sup>CXCR5<sup>+</sup> T cells producing IL-21 were compared between patients with AS (n=54; some patients with AS failed the test and were not included in statistical analysis) and HC (n=20). IL, interleukin; CD, cluster of differentiation; CXCR5, C-X-C chemokine receptor type 5; NS, no significant differences; HC, healthy controls; AS, ankylosing spondylitis; SSC, side scatter data; FSC, forward scatter data.

data suggest that different phenotypes of cTfh cells may have variable functions in regulating the differentiation of B cells during AS development.

## Discussion

Tfh cells have been recognized in recent years as a crucial regulator of GC formation, B cell development and long-term humoral memory generation (25-27), and have a significant role in the pathogenesis of autoimmune diseases. Previous studies (5,6) have characterized the frequency of circulating Tfh cells in AS. However, the mechanisms by which cTfh cells and associated molecules regulate B cell differentiation in humans remain largely unknown. In the present study, it was observed that the frequency of peripheral blood CD4<sup>+</sup>CXCR5<sup>+</sup> cTfh cells was significantly higher in patients with AS than in HC. Furthermore, the frequency of CD4<sup>+</sup>CXCR5<sup>+</sup> cTfh cells was positively correlated with BASDAI values in patients with AS. These results improve upon those of previous studies by Xiao *et al* (5) and Wu *et al* (6), which did not reveal the correlation of CD4<sup>+</sup>CXCR5<sup>+</sup> cTfh cells with BASDAI values in patients with AS.

ICOS and PD-1 are expressed by Tfh cells (28) and are closely associated with the function of Tfh cells (29-31). ICOS is known as a positive regulator of Tfh cells and previous studies using a mouse model of AS indicated that ICOS

overexpression induces overproduction of CD4<sup>+</sup>CXCR5<sup>+</sup> Tfh cells and exuberant GC responses, and promotes antibody production (32,33), whereas PD-1 is a potent negative regulator of humoral immune responses (31,34). The present study demonstrated increased percentages of CD4<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>+</sup> T and CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>+</sup> T cells in patients with AS compared with those in HC. According to BASDAI values, which are important for the evaluation of AS disease activity, patients were categorized into high activity and low activity AS groups. It was observed that higher percentages of CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>+</sup> T cells were present in the high activity AS group compared with the low activity AS group. Conversely, lower percentages of CD4<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>+</sup> T cells were detected in the high activity AS group compared with the low activity AS group. However, differences between these two comparisons were not significant. As expected, there was no correlation between BASDAI values and the percentages of CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>+</sup> T cells or CD4<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>+</sup> T cells. These data suggest that high percentages of CD4<sup>+</sup>CXCR5<sup>+</sup> cTfh cells and expression of ICOS and PD-1 may be associated with the pathogenesis of AS; however, further research is required to elucidate the precise function of ICOS and PD-1 in AS.

Subsequently, to better understand the role of Tfh in the pathogenesis of AS, the main effector cytokines of Tfh cells were investigated, including IL-21, which exists with IL-6, regulates the differentiation of Tfh cells and induces B cell proliferation

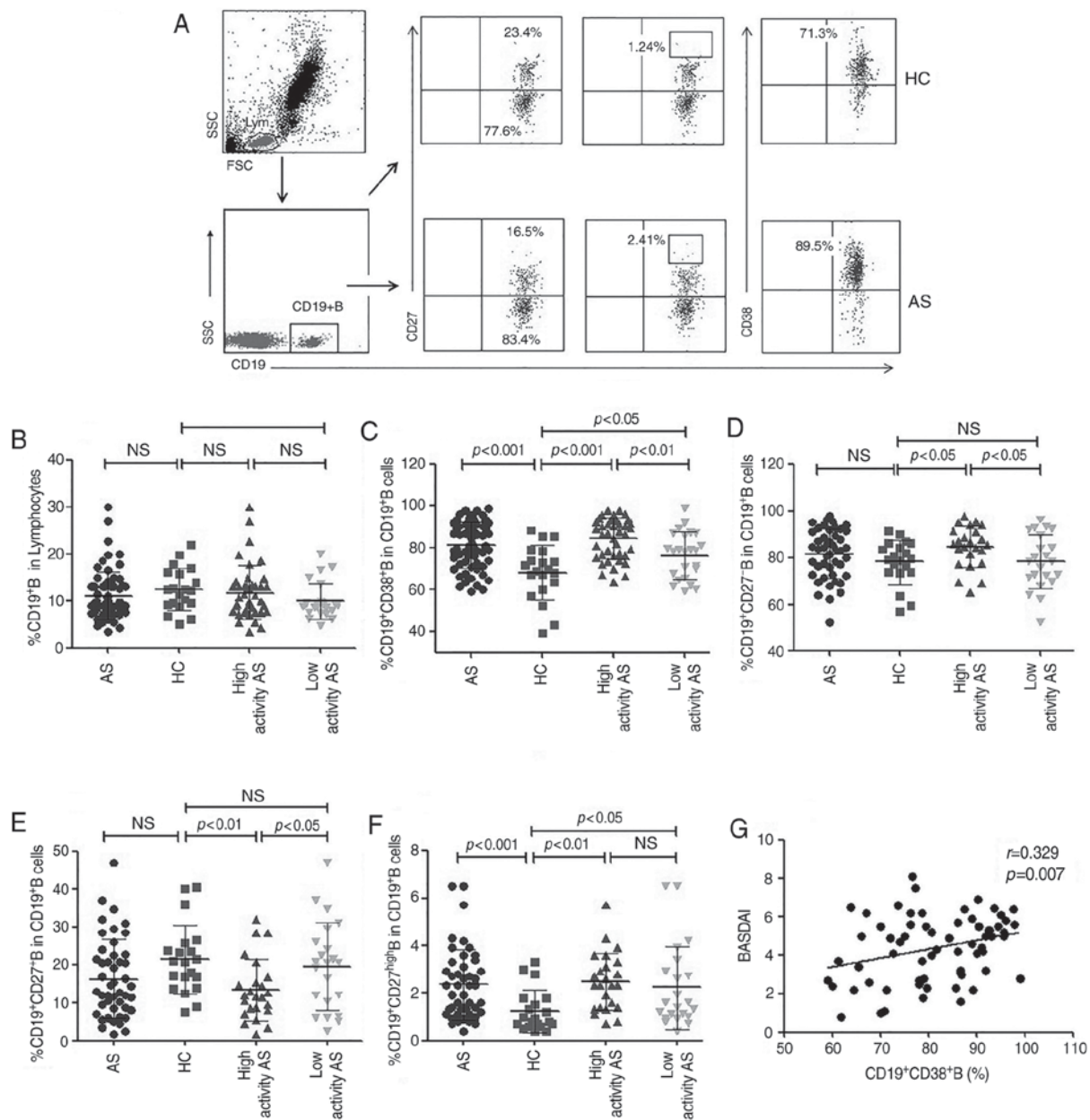


Figure 3. Flow cytometry analysis of different subtypes of B cells. Peripheral blood in duplicate was stimulated with anti-CD19, anti-CD27 and anti-CD38 antibodies. After gating CD19+B cells, frequencies of CD19+CD27<sup>naïve</sup> B cells, CD19+CD27<sup>memory</sup> B cells, CD19+CD27<sup>high</sup> plasmablast B cells and CD19+CD38<sup>+</sup> antibody-secreting B cells were analyzed. (A) Representative flow cytometry analysis. Percentages of (B) CD19+B and (C) CD19+CD38<sup>+</sup> antibody-secreting B cells were compared between patients with AS (n=65) and HC (n=20). Percentages of (D) CD19+CD27<sup>naïve</sup> B cells, (E) CD19+CD27<sup>memory</sup> B cells and (F) CD19+CD27<sup>high</sup> plasmablast B cells were compared between patients with AS (n=46) and HC (n=20). (G) Correlation analysis (n=65). CD, cluster of differentiation; NS, no significant differences; HC, healthy controls; AS, ankylosing spondylitis; SSC, side scatter data; FSC, forward scatter data; BASDAI, Bath AS Disease Activity Index.

and class switch recombination (24,35). Xiao *et al* (5) previously observed a higher frequency of peripheral blood IL-21 positive Tfh cells in patients with AS—a result which the present findings expand upon. The present study demonstrated that there were significantly higher percentages of CD3<sup>+</sup>CD8<sup>+</sup> IL-21<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>CXCR5<sup>+</sup>IL-21<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup>CXCR5<sup>+</sup>IL-21<sup>+</sup> T cells in patients with AS compared with HC. Notably, the percentages of CD3<sup>+</sup>CD8<sup>+</sup> IL-21<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>CXCR5<sup>+</sup>IL-21<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup>CXCR5<sup>+</sup>IL-21<sup>+</sup> T cells were positively correlated with BASDAI values in patients with AS. These findings suggest that regardless of cellular sources of IL-21, they may participate in the development of AS.

The activation and differentiation of B cells require help from Tfh cells. Different subtypes of B cell express unique profiles of immunomodulatory factors and thereby evolve distinct functions. CD19+CD27<sup>naïve</sup> B cells display no maturity and are defined as naïve B cells (36). Upon gain of expression of CD27 (CD19+CD27<sup>+</sup> B cells), the cells become activated memory B cells (37). CD19+CD27<sup>high</sup> B cells are defined as plasmablasts, whereas upon the expression of CD38, the cells lead to the antibody-secreting phenotype (CD19+CD38<sup>+</sup>) (17,18). In the present study, it was demonstrated that the percentages of CD19+CD27<sup>high</sup> plasmablasts and CD19+CD38<sup>+</sup> antibody-secreting B cells were increased

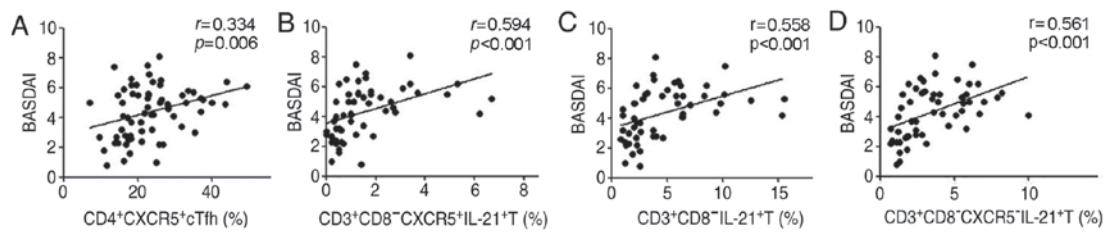


Figure 4. Association between the percentages of different phenotypes of cTfh and BASDAI values in patients with AS. (A) The percentage of CD4<sup>+</sup>CXCR5<sup>+</sup> cTfh cells is positively correlated with BASDAI values in patients with AS (n=65) using Spearman's rank test. (B) The percentage of CD3<sup>+</sup>CD8<sup>+</sup>CXCR5<sup>+</sup>IL-21<sup>+</sup> T cells is positively correlated with BASDAI values in patients with AS (n=54) using Spearman's rank test. (C) The percentage of CD3<sup>+</sup>CD8<sup>+</sup>IL-21<sup>+</sup> T cells is positively correlated with BASDAI values in patients with AS (n=54) using Spearman's rank test. (D) The percentage of CD3<sup>+</sup>CD8<sup>+</sup>CXCR5<sup>+</sup>IL-21<sup>+</sup> T cells is positively correlated with BASDAI values in patients with AS (n=54) using Spearman's rank test. cTfh, circulating follicular helper T cells; BASDAI, Bath AS Disease Activity Index; AS, ankylosing spondylitis; CD, cluster of differentiation; CXCR5, C-X-C chemokine receptor type 5; IL, interleukin.

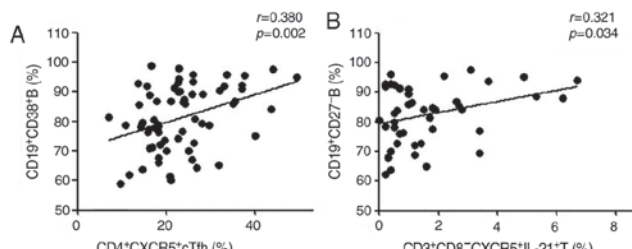


Figure 5. Association between the percentages of cTfh and B cells in patients with AS. (A) The percentage of CD4<sup>+</sup>CXCR5<sup>+</sup> cTfh cells is positively correlated with the frequency of CD19<sup>+</sup>CD38<sup>+</sup> antibody-secreting B cells in patients with AS (n=65) using Pearson's rank tests. (B) The percentage of CD3<sup>+</sup>CD8<sup>+</sup>CXCR5<sup>+</sup>IL-21<sup>+</sup> T cells is positively correlated with the frequency of CD19<sup>+</sup>CD27<sup>+</sup> naïve B cells in patients with AS (n=44) using Pearson's rank tests. cTfh, circulating follicular helper T cells; AS, ankylosing spondylitis; CD, cluster of differentiation; CXCR5, C-X-C chemokine receptor type 5; IL, interleukin.

in patients with AS, relative to HC, although there was no significant difference in the frequency of CD19<sup>+</sup> B cells, CD19<sup>+</sup>CD27<sup>+</sup> naïve B cells, and CD19<sup>+</sup>CD27<sup>+</sup> memory B cells between patients with AS and HC. These results suggest that, following antigen stimulation, the distribution of B cell subtypes was changed and the proportions of the plasmablasts and antibody-secreting B cells were highly increased compared with other B cell subtypes in patients with AS. This was likely due to a higher presence of plasmablasts and antibody-secreting B cells following the high differentiation of bone marrow stem cells in order to produce more antibodies against a foreign antigen. The present findings were partially consistent with a previous study by Lin *et al* (15), which demonstrated that, compared with controls, the percentages of CD19<sup>+</sup> B cells and subsets (CD19<sup>+</sup>CD27<sup>+</sup>, CD19<sup>+</sup>CD27<sup>dim</sup> and CD19<sup>+</sup>CD27<sup>high</sup>) were not significantly different in patients with AS. These disparities may be due to a number of reasons, including varying genetic backgrounds, disease duration and cohort size.

The present study also demonstrated that, compared with the low activity AS group, the high activity AS group exhibited increased percentages of CD19<sup>+</sup>CD38<sup>+</sup> antibody-secreting B cells and CD19<sup>+</sup>CD27<sup>+</sup> naïve B cells, but a decreased percentage of CD19<sup>+</sup>CD27<sup>+</sup> memory B cells. These results indicate that there may be an association between B cell subtypes and BASDAI values. However, the present results have only revealed a significant positive correlation between

the percentage of CD19<sup>+</sup>CD38<sup>+</sup> antibody-secreting B cells and BASDAI values in patient with AS, which is in accordance with a previous study (38). This suggests that particular B cell subtypes may be associated with different stages of AS disease pathogenesis and the percentage of CD19<sup>+</sup>CD38<sup>+</sup> antibody-secreting B cells may be a potential biomarker to evaluate the disease activity of AS.

It has been reported previously that Tfh cells are able to regulate B cell activation and promote B cell maturation in autoimmune disease (8,20). In the present study, it was observed that the percentage of CD4<sup>+</sup>CXCR5<sup>+</sup> cTfh cells was positively correlated with the percentage of CD19<sup>+</sup>CD38<sup>+</sup> antibody-secreting B cells in patients with AS. The percentage of CD3<sup>+</sup>CD8<sup>+</sup>CXCR5<sup>+</sup>IL-21<sup>+</sup> T cells was also correlated positively with the frequency of CD19<sup>+</sup>CD27<sup>+</sup> naïve B cells in patients with AS. These findings may be a clinical display of a previous *in vitro* finding by Morita *et al* (12), which demonstrated that culturing naïve B cells with CXCR5<sup>+</sup>CD4<sup>+</sup> T cells yielded higher numbers of CD38<sup>+</sup>CD19<sup>lo</sup> cells than with CXCR5<sup>+</sup>CD4<sup>+</sup> T cells. This indicated that CXCR5<sup>+</sup>CD4<sup>+</sup> T cells were more efficient at inducing naïve B cells to differentiate into CD38<sup>+</sup>B cells. In addition, the study by Morita *et al* (12) also demonstrated that blood CXCR5<sup>+</sup>CD4<sup>+</sup> T cells secreted IL-21 upon contact with naïve B cells. High percentages of CXCR5<sup>+</sup>CD4<sup>+</sup> T cells secrete more IL-21 following interaction with naïve B cells in patients with AS, as the increased IL-21 promotes naïve B and memory B cells differentiation into antibody-secreting B cells (39). This may explain why antibody-secreting B cell levels markedly increased and more naïve B cells were released by bone marrow. Furthermore, this may be the underlying reason for the significant positive correlations between cTfh and B subtypes. These findings support the hypothesis that Tfh cells may regulate the distribution of B cell subtypes via different functional molecules; however, further research is required to elucidate the precise molecular and immunological mechanism.

In conclusion, the present findings suggest that the percentages of cTfh cells and activated B cell subtypes are significantly increased and positively correlated with disease activity in patients with AS, and the percentage of cTfh cells is positively correlated with particular B cell subtypes. The limitations of the present study, which must be considered when interpreting these results, include the relatively small sample size and the lack of cell culture experiments on cTfh cells and B cells. Further studies are required to address the

relationship between circulating Tfh cells and B cell subtypes in patients with AS.

### Acknowledgements

The authors would like to thank Dr Fred Bogott at the Austin Medical Center (Austin, MN, USA) and Dr Joshua Liao at Hormel Institute of University of Minnesota (Austin, MN, USA) for their English language editing of this manuscript.

### Funding

No funding was received.

### Availability of data and materials

The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

### Authors' contributions

LM and SQL designed and directed the research. SQL performed the experiments, analyzed and interpreted data, and drafted the manuscript. DSW collected the experimental data and SWS collected the clinical data. All authors read and approved the final for publication.

### Ethics approval and consent to participate

All patients provided written informed consent prior to their inclusion in the present study and the experimental protocol was approved by the institutional ethics committee of Guizhou Medical University Hospital (Guiyang, China).

### Consent for publication

All patients provided written informed consent prior to their inclusion in the present study.

### Competing interests

The authors declare that they have no competing interests.

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