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Original Article

Analysis of apoptosis and cell cycle of lymphocyte subsets in peripheral blood of patients with ankylosing spondylitis

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Abstract: Ankylosing spondylitis (AS) is an autoimmune disease. Changes of immune cells are associated with AS disease progression. However, apoptosis and cell cycle of peripheral blood lymphocyte subsets in AS patients remains unclear. Peripheral blood from patients with active and stable AS, and healthy controls were collected for isolation of peripheral blood mononuclear cells (PBMCs) and naive T cells (Tn). Tn cells were cultured *in vitro* for analysis of cell proliferation by MTT assay, apoptosis, cell cycle, CD4+, Treg, and CD8+ T cells by flow cytometry, as well as the secretion of interferon-γ (IFN-γ), interleukin-4 (IL-4) and interleukin-10 (IL-10) by ELISA. Compared with the control group, proliferation of Tn cells in active and stable AS patients was significantly increased, cell apoptosis was decreased, cell cycle G2/M ratio was decreased, proportion of CD4+ T cells was increased, proportion of Treg cells was decreased, secretion of IFN-γ was increased and secretion of IL-4 and IL-10 were decreased ($P<0.05$). The changes in the above parameters were more significant in active AS patients compared with the stable AS patients ($P<0.01$). The proliferation of Tn cells in AS patients increased. Apoptosis of Tn cells in AS patients decreased. The cell cycle is changed significantly. The classification of lymphocyte subsets is changed significantly, with more significant changes in the active phase, which was closely related to AS progression.

Keywords: Ankylosing spondylitis, peripheral blood lymphocytes, apoptosis, cell cycle, Treg

Introduction

Ankylosing spondylitis (AS) is a chronic inflammatory autoimmune disease. It is one of the most common immune rheumatic diseases. It affects men more often than women and is mainly found in young adults under 40 years of age and whom have family heredity [1, 2]. The rheumatoid factor in AS was a negative result, involving multiple loci, and it was confirmed that AS and HLA-B27 were positively correlated [3]. The occurrence of AS has genetic characteristics, and the incidence in Asian people is higher than that in Caucasians [4]. AS mainly affects the spine, ankle joints and other central limb joints. It is a form of spinal arthritis and is a progressive inflammatory disease. The main clinical manifestations include typical inflammatory back pain, and progressive accumulation of peripheral arthritis in tendons and ligaments. Inflammation of the tendon develops into buttocks pain, hip pain, low back pain, and limited spinal activity [5]. The pathological features of AS are chronic inflammatory changes involving tendons, ligaments, ankles, etc., leading to increased expression of inflammatory factors in the serum. It has core pathological links from inflammation to bone destruction and new bone formation [6, 7]. AS not only causes joint stiffness, fibrosis and ossification of the intervertebral disc and surrounding tissues, but also accumulates in multiple tissues such as peripheral joints, intestines, lungs, and muscles [8]. AS is commonly concealed as opposed to being overt. The progress of the disease is usually slow, and the disability rate is high, which brings great suffering and economic burden to patients, as it becomes one of the public health problems in the world [9]. Although there have been studies confirming that the pathogenesis of AS appears to involve genetic, immune, environmental and other factors, it has not been fully elucidated [10]. In several autoimmune diseases, abnormalities in im-

mune cells appear to be one of the most are important pathogenic factors [11]. CD4+ T cells can differentiate into Th1 cells, Th2 cells, etc. through secreted cytokines, and then can play a role in promoting phagocytic-mediated anti-infective immunity [12, 13]. CD4+ T cells also proliferate and differentiate into B lymphocytes in normal physiological conditions, produce antibodies, and participate in humoral immune responses. CD4+ T cells are confirmed to be involved in AS [14]. Treg cells are involved in the development of a variety of immune-related diseases, particularly autoimmune disease [15]. In the presence of T cell antigen receptor (TCR) stimulation and corresponding cytokines, naïve T cells (Tn cells) can be induced to differentiate into regulatory T cells and Th1 and Th2 cells, etc. [16]. This study focused on analyzing the proliferation, apoptosis and cell cycle of lymphocyte subsets in the peripheral blood of patients with AS in different time periods, aiming to provide a theoretical basis for the pathogenesis of AS.

Materials and methods

General information

From January 2017 to December 2017, 70 patients with active AS were enrolled in the Department of Rheumatology, including 52 males and 18 females, aged 22-39 years, with an average age of 25.0±8.2 years. The criteria for diagnosis are based on the AS diagnosis basis established by the American College of Rheumatology (ACR) [17]. The diagnosis was made by at least 2 experienced experts who specialize in rheumatology and immunology. The inclusion criteria: AS that meets either clinical criteria or imaging criteria, or meets both criteria. Clinical criteria included inhibition of lumbar motion, low back pain, morning stiffness for at least 3 months that cannot be alleviated by rest, and reduced thoracic activity. Imaging diagnostic criteria include an arthritis grading scale, and whether was unilateral III-IV or bilateral ≥II [17]. Exclusion criteria: recent history of infection, association with vital organ damage such as tumor in liver or kidney, chronic inflammatory disease, severe cardiovascular and cerebrovascular diseases, abnormal blood and endocrine system diseases, other rheumatic diseases, current pregnancy or lactating. AS patients with active disease were enrolled according to the BASDAI score. AS patients with a BASDAI score greater than 3 were grouped into the AS disease activity group [17]. In the healthy control group, 35 volunteers who underwent physical examination at the same time were enrolled. Those volunteers did not have any family history nor chronic disease history of autoimmune diseases. There were 23 males and 12 females, aged 20-40 years, mean age (25.0±8.5) years old included in the study. The study was approved by the Medical Ethics Committee of the 80th Army Hospital of the Chinese People's Liberation Army and informed consent was signed by all the participants.

Main reagents and instruments

Lymphocyte separation solution was purchased from Tianjin Haoyang Biological Co. Ltd. (China). RPMI 1640 complete medium was purchased from Hyclone (USA). SYTOX Green, rabbit anti-human Perep-CD4 mAb, rabbit anti-human APC-CD25 mAb, rabbit anti-human phycoerythrin cyanin-7 (PE-Cy7)-CD45RA mAb, rabbit anti-human anti-CD3 monoclonal antibody were purchased from BD (USA). Anti-CD3/CD28 magnetic beads were purchased from Life Technology (USA). The Annexin V-PI kit was purchased from BD (USA). The cell cycle assay kit was purchased from BD (USA). IFN-γ, IL-4, IL-10 ELISA kits were purchased from R&D (USA). The SpectraMax iD5 microplate reader was purchased from Molecular Devices (USA). The Melody C6 flow cytometer and the FACS Aria II flow cytometer were purchased from BD (USA). Other commonly used reagents were purchased from Shanghai Shenggong Biological Co., Ltd (China).

PBMCs and Tn cells and Treg cell sorting

Peripheral venous blood of 20 patients in each stage of AS and healthy controls was anticoagulated with heparin sodium. After adding 20 mL PBS buffer and 20 mL lymphocyte separation solution, blood was centrifuged at 2000 rpm for 20 min to obtain PBMCs. We then resuspended it in RPMI 1640 complete medium followed by addition of SYTOX Green, PerCP-CD4 mAb, APC-CD25 and phycoerythrin cyanin-7 (PE-Cy7)-CD45RA and incubation for 20 min at 4°C in the dark. After washing with 10 mL of PBS buffer, the supernatant was discarded.

Two mL of RPMI 1640 complete medium was added, and after filtration through a sieve, Tn cells and Treg cells were sorted by flow cytometry.

Tn cell proliferation assay

After 48 hours of cell culture, cells were digested and counted, and seeded into 96-well plates at a density of 3000 cells/well. Five replicate wells were designed for each group, and 20 μl of 5 g/L MTT solution was added to each well. After 4 hours of incubation, the supernatant was completely removed, 150 μl/well of DMSO was added, and was shaken for 10 min. After the purple crystals were fully dissolved, the absorbance (A) value was measured at a wavelength of 570 nm by a microplate reader to calculate the cell proliferation rate.

Flow cytometry analysis of Tn cell apoptosis

Flow cytometry was used to analyze the apoptosis rate of Tn cells in each group. 1×106 Tn cells were resuspended in RPMI 1640 complete medium, seeded in 24-well cell culture plates. One:one Tn cells and magnetic beads were used. Anti-CD3/CD28 magnetic beads were added. The cells were harvested after 24 hours, washed with Annexin V Binding Buffer, resuspended in 100 μL of washing solution followed by addition of Annexin V and PI and incubation for 15 min in the dark. After washing with Annexin V Binding Buffer, apoptosis of Tn cells was detected by flow cytometry.

Flow cytometry analysis of Tn cell cycle

The cell cycle of Tn cells was analyzed by flow cytometry. Cells in logarithmic growth phase were seeded on a 6-well cell culture plate at a density of 4×10^5/mL. After the overnight culture, cell medium was washed once with 1 ml of PBS buffer. After centrifugation at 800 g for 6 min, cells were resuspended in pre-cooled absolute ethanol for fixation at 4°C in the dark overnight. After that, cells were centrifuged at 800 g for 10 min, washed twice with PBS. Cells then were incubated with PBS buffer containing 100 unit/ml RNaseA at 37°C for 30 min in the dark followed by addition of 2 mg/ml PI. Subsequent analysis of cell cycle was detected by flow cytometer.

Flow cytometric analysis of lymphocyte subsets

The cell culture solution of each group of cells was discarded, and the adherent cells were washed once with pre-cooled PBS, centrifuged at 1000 g for 5 min and resuspended in 1× Binding buffer followed by incubation with 5 μl of anti-CD3 and 5 μl of anti-CD8 antibody, anti-CD4 antibody, anti-CD25, and Foxp3 antibody for 5 min. Then, lymphocyte subsets were detected by flow cytometry.

Specimen collection

Five ml of fasting peripheral venous blood of AS patients and healthy controls were collected into heparin sodium anticoagulant tube, centrifuged at 2000 rpm for 10 min. The supernatant was placed in a freezer at -20°C for later use.

ELISA analysis of level of cytokines

The expression of IFN-γ, IL-4 and IL-10 in serum of each group was detected by ELISA according to the ELISA kit purchased from R&D (USA). The optical density value (OD value) of each well was measured by a microplate reader, and a standard curve was prepared according to the OD value and the corresponding concentration was detected by the standard product. The corresponding sample concentration was then calculated.

Statistical analysis

All data was processed by SPSS 22.0 software. Measurement data was described by mean ± standard deviation (SD). One-way ANOVA was used for comparison of multiple groups of samples. The student t-test was used for comparison between the two groups. The test level is $\alpha=0.05$. $P<0.05$ was a statistically significant difference.

Results

Analysis of classification of lymphocyte subsets in AS patients

Flow cytometry was used to analyze the lymphocyte subsets in patients with stable and active AS. The results showed that compared with control group, the proportion of CD4+ T cells in active and stable AS patients was sig-

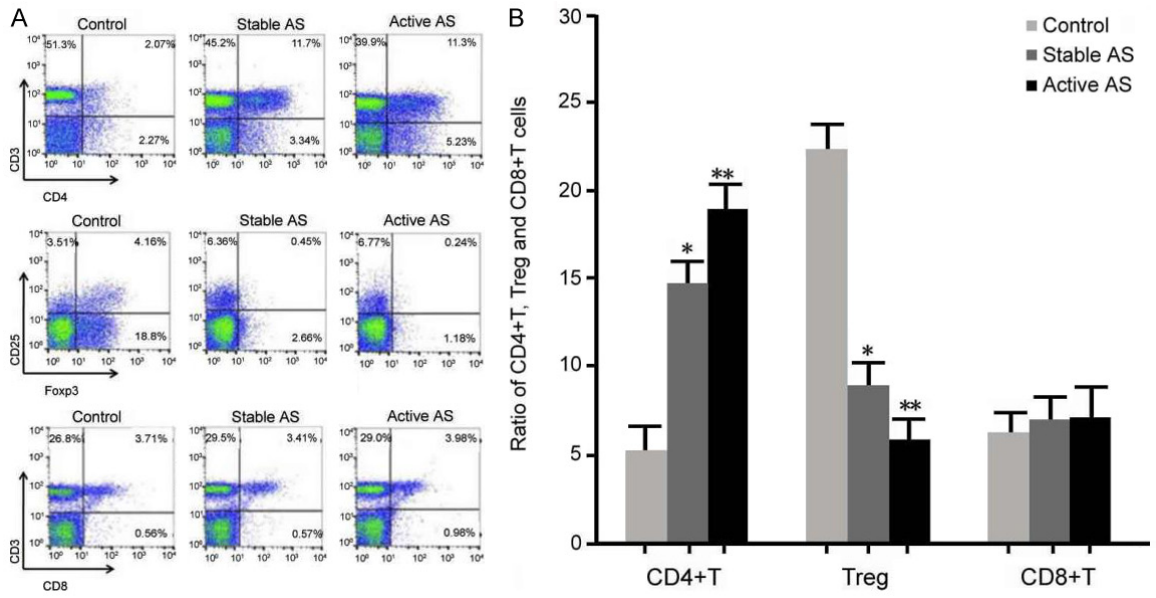

Figure 1. Analysis of classification of lymphocyte subsets in AS patients. A. Flow cytometry analysis of lymphocyte subsets in stable and active AS patients; B. Statistical analysis of lymphocyte subsets. Compared with healthy controls, *P<0.05; **P<0.01.

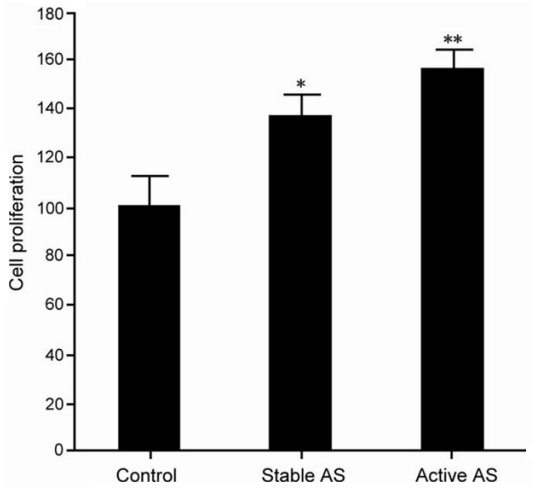

Figure 2. Changes in lymphocyte Tn cell proliferation in AS patients. Compared with healthy control group, *P<0.05; **P<0.01.

nificantly increased, the proportion of Treg cells was decreased (P<0.05) without any noticeable changes of CD8+ T cells. The changes of the above parameters in AS patients were more significant (P<0.01) (**Figure 1**).

Changes of lymphocyte Tn cell proliferation in AS patients

MTT assay was used to analyze the proliferation of lymphocyte Tn cells in patients with stable and active AS. Compared with the control group, the proliferation of Tn cells in active and stable AS patients was significantly increased (P<0.05), and the changes in active AS patients were more significant (P<0.01) (**Figure 2**).

Apoptosis of lymphocyte Tn cells in AS patients

Flow cytometry was used to analyze the changes of lymphocyte Tn cell apoptosis in patients with stable and active AS. Compared with control group, the apoptosis of Tn cells in the active and stable AS patients decreased, the difference was statistically significant (P<0.05). More changes were observed in active AS patients (P<0.01) (**Figure 3**).

Analysis of cell cycle changes of lymphocyte Tn cells in AS patients

Flow cytometry was used to analyze the cell cycle changes of lymphocyte Tn cells in patients with stable and active AS. Compared with control, the G2/M ratio of Tn cells in active and stable AS patients decreased and the difference was statistically significant (P<0.05) with more changes in active AS patients (P<0.01) (**Figure 4**).

Changes of lymphocyte secretion cytokines in AS patients

ELISA was used to analyze the changes of lymphocyte secretion cytokines in patients with

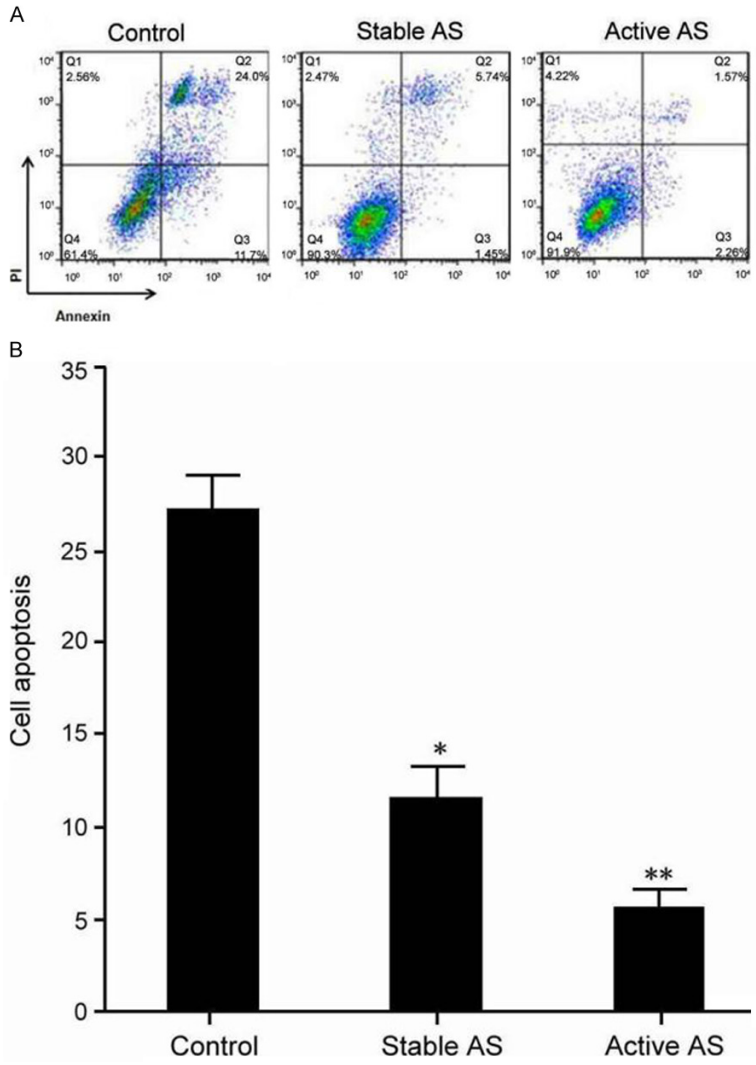

Figure 3. Apoptosis of lymphocyte Tn cells in AS patients. A. Flow cytometry was used to analyze lymphocyte apoptosis in stable and active AS patients; B. Statistical analysis of lymphocyte apoptosis. Compared with healthy controls, *P<0.05; **P<0.01.

stable and active AS. The results showed that compared with the control group, the secretion of cytokine IFN-γ was increased and the secretion of IL-4 and IL-10 was decreased in the active and stable AS patients, and the differences were statistically significant (P<0.05). More changes were found in AS patients (P<0.01) (**Figure 5**).

Discussion

AS is one of the most common type of spinal arthritis. Its pathogenesis has not been fully elucidated, yet it is supported by numerous research that it is closely related to lymphocyte expression and dysfunction, especially peripheral blood T lymphocytes [18]. Therefore, this study focused on the peripheral blood lymphocyte subsets in patients with stable and active AS. The results suggest that compared with the healthy control group, the proportion of CD4+ T cells in the active and stable AS patients was increased, the proportion of Treg cells was decreased, and the proportion of CD8+ T cells did not change; indicating that CD8+ T cells show more stable distribution in the presence and progression of AS, and changes in the distribution of CD4+ T cells and Treg cells are involved in the development of AS.

Naive T cells (Tn) mainly differentiate into CD4+ T cells and Treg cells, so this study further analyzed the proliferation, apoptosis and cell cycle changes of Tn, and then clarified the changes of AS cell subsets. This study confirmed that compared with healthy controls, active and stable AS patients showed increased Tn cell proliferation, decreased apoptosis, decreased cell cycle G2/M ratio, increased CD4+ T cell ratio, decreased Treg cell ratio, increased secretion of IFN-γ, and decreased secretion of IL-4 and IL-10, with more changes in active AS patients. Studies have shown that CD4+ T cells and Treg cells are abnormally distributed and expressed in AS. CD4+ T cells further differentiate into helper T cells, and can differentiate into Th1 and Th2, in the occurrence and development of autoimmune diseases. This plays an important role, along with the imbalance of cytokine secretion by Th1 and Th2 cells, which might be an important factor in autoimmune diseases [19, 20]. Th1 and Th2 cells are in equilibrium under normal conditions, maintaining the homeostasis of the body. Th1/Th2 imbalance can be found in autoimmune diseases such as irritable enteritis, asthma, and rheumatoid arthritis. The Th1 cytokine IFN-γ antagonizes the release of the Th2 secreting cytokine IL-4,

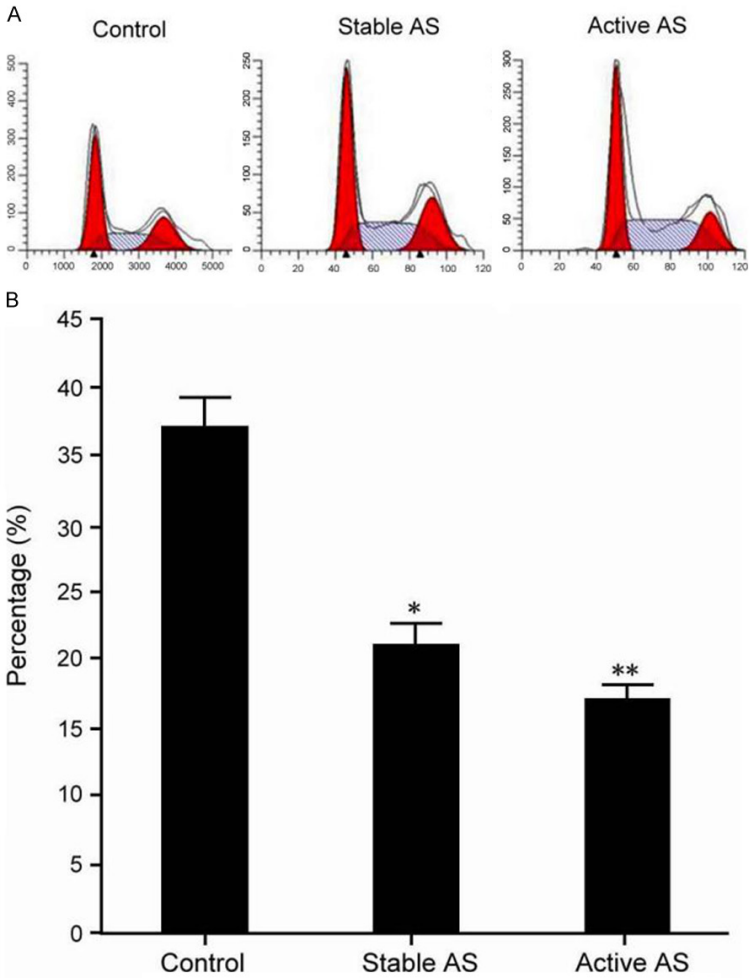

Figure 4. Analysis of cell cycle changes of lymphocyte Tn cells in AS patients. A. Flow cytometry analysis of lymphocyte cell cycle changes in stabilizer and active AS patients; B. Statistical analysis of lymphocyte cell cycle changes. Compared with healthy controls, *P<0.05; **P<0.01.

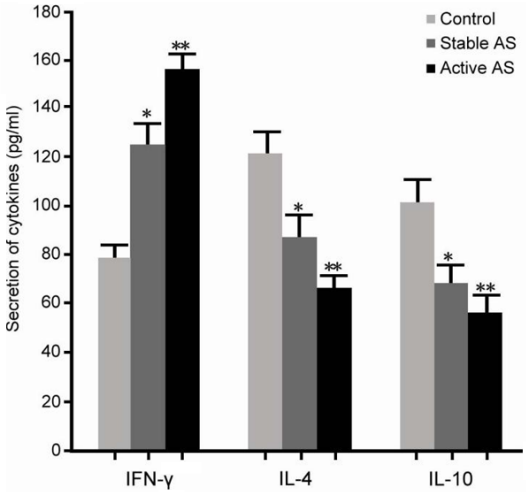

Figure 5. Changes in lymphocyte secretion cytokines in AS patients. Compared with healthy control group, *P<0.05; **P<0.01.

which in turn inhibits the Th2 response [21]. The results of this study confirmed that CD4+ T cells in AS patients were further differentiated into Th1 cells, which secreted IFN-γ and inhibited Th1, leading to the development of AS. Treg cells mainly play an immunosuppressive role, which can inhibit the immune cells directly or by secreting the cytokine IL-10 [22]. The abnormal number and function of Treg cells can lead to the occurrence of tumors and autoimmune diseases. There are several contradictions about the expression of Treg in AS. It is believed that the expression ratio of Treg cells in AS patients is increased, and some studies have suggested that the expression is decreased [23-25]. However, this study confirmed that the proportion of Treg cells was decreased, and the expression of cytokine IL-10 was decreased. The change in active phase was more significant, indicating that Tn cell proliferation was increased and apoptosis was decreased, which mainly differentiated into CD4+ T cells with a predominance of Th1 cells, whereas, few Tn cells differentiated into Treg cells, leading to a decrease in the proportion of Treg cells and a decrease in the secretion of cytokines, which causes a decrease in immunosuppressive effects, thereby inducing the occurrence and development of AS. In further research, we propose an area to focused on is to analyze the regulatory mechanism of peripheral blood lymphocytes in AS in order to provide a basis for elucidating the pathogenesis of AS.

Conclusion

The lymphocyte subsets in AS patients are significantly changed, mainly because the proportion of CD4+ T cells is increased, while the proportion of Treg cells is decreased, and the proportion of CD8+ T cells remains is unchanged. Tn cell proliferation increased, apoptosis

decreased, with changes of cell cycle more significant in active AS, suggesting that it is closely related to the progression of AS.

Disclosure of conflict of interest

None.

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