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Effect of abnormal activated B cells in patients with ankylosing spondylitis and its molecular mechanism

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Abstract. – OBJECTIVE: Ankylosing spondylitis (AS) is a common clinical autoimmune disease, the pathogenesis of which, however, is not yet elucidated. In this study, we aim to explore the effect of B cells in the development and progression of AS and its underlying mechanism.

PATIENTS AND METHODS: B cells were isolated from peripheral blood of AS patients and normal controls. Surface expression of CD40 in B cells was detected by flow cytometry. Expressions of downstream genes in MAPK pathway were detected by Western blot. Moreover, IL-10 expressions in peripheral blood of AS patients and normal controls were detected by ELISA.

RESULTS: No difference was found in the surface expression of CD40 in B cells between AS patients and normal controls. However, CD40 expression was inhibited after B cells in peripheral blood were specifically stimulated by lipopolysaccharide (LPS) *in vitro*. Abnormal activated B cells, dysregulated p38 expressions and decreased serum expressions of IL-10 were also observed in AS patients.

CONCLUSIONS: Abnormal surface expression of CD40 inn B cells of AS patients may lead to abnormal activation of B cells, thereby interfering the p38 MAPK pathway and reducing the IL-10 secretion.

Key Words:
Ankylosing spondylitis, MAPK pathway, CD40, IL-10.

Introduction

Ankylosing spondylitis (AS) is a type of arthritis in which there is a long-term inflammation of the joints of the spine. AS is closely related to genetic factors, microbial infections, endocrine disorders, and immune dysregulation[1]. Typically, sacroiliac joints, spine, shoulder, and hip are affected[2]. Clinical manifestations of AS are mainly joint pain, joint swelling, spinal deformity, and joint stiffness. Heart disease, eye and ear lesions and other extra-articular manifestations may also occur in AS patients[3]. Clinical diagnosis of AS is usually based on clinical symptoms, imaging, and serological examination[4]. So far, AS is widely distributed worldwide, and its incidence has been increased in recent years. Unfortunately, there is no cure for AS. Current treatments of AS can only improve symptoms and prevent worsening[3].

Attenuated immune tolerance in patients with autoimmune diseases leads to abnormal activation of B cells. Briefly, autoantigen is recognized as an external antigen presenting to T cells, which in turn stimulate B cells to produce pathogenic autoantibodies, thereby damaging the body tissue[5]. Since AS is closely related to immune regulation, researches on the immune response to AS are well recognized. Previous researches have indicated that surface expressions of CD154 in T cells of AS patients are much higher than those of healthy people, indicating that the abnormal activated T cells are involved in the development and progression of AS. Currently, there are many studies focused on the correlation between T cells and AS. However, the relationship between B cells and AS pathogenesis remains unclear. In the present study, we aim to explore the relationship between B cells and AS.

Abnormal activated T cells are involved in the AS pathogenesis. The interaction of CD40 ligands (CD40L, CD154) with surface expression of CD40 in T cells is the secondary signal to activate T and B cells, thus regulating the immune response. Activated B cells are primarily associated with CD40 molecule on B cells surface[6]. Over-activated CD40 leads to increased expressions of inflammatory cytokines and adhesion molecules[7], which may cause some chronic in-

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flammatory diseases such as rheumatoid arthritis (RA)[8,9], graft versus host disease[10-12], and atherosclerosis[13-15]. Therefore, it is of great importance to investigate the effect of CD40 expression on the AS pathogenesis.

Patients and Methods

Patients

A total of 38 subjects were enrolled in this study, including 18 AS patients (10 male and 8 female) aged from 25 to 58 years, and 20 normal controls (11 male and 9 female) aged from 26 to 60 years. This study was approved by the Ethics Committee of the Affiliated Hospital of Weifang Medical University. Signed written informed consents were obtained from all participants before the study.

Extraction of Human Peripheral Blood Mononuclear Cells (PBMCs)

Isolation of cells was performed using PBMCs isolation methods with slight manipulation. Briefly, 2 mL of peripheral blood was obtained and diluted in 2 mL of saline. Peripheral blood was then collected in cell preparation tubes containing 2 mL of lymphocyte separation solution. Diluted blood was slowly added to the separation solution, followed by centrifugation at 800 g for 25 min. Buffy coat was collected and centrifuged again at 250 g for 10 min. The obtained precipitates were PBMCs.

Isolation and Culture of B Cells in Peripheral Blood

PBMCs were re-suspended in magnetic bead sorting solution (BD Biotech, Franklin Lakes, NJ, USA) and B cells were obtained by negative sorting. B cells in human peripheral blood were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA) and maintained in a 5% CO_2 incubator at 37°C.

Flow Cytometry

Cells were centrifuged, washed twice with pre-cooled phosphate-buffered saline (PBS) and incubated with CD-FITC antibody and CD40-PE antibody in the dark for 30 min. 200 μL of PBS was used for re-suspending the cell precipitat ion. Finally, the positive expression of CD40-PE was detected by flow cytometry.

Quantitative Real-Time PCR (qRT-PCR)

Cellular mRNAs were extracted by TRIzol reagent, and then, reversely transcribed to cDNAs. Each sample was repeatedly performed for 3 times. Primers used in this study were as follows: Human IL-10 forward primer: 5'-GGTTGCCAAGCCTTATCGGA-3'; Human IL-10 reverse primer: 5'-ACCTGCTCCACTGCCTTGCT-3'; Human p38 forward primer: 5'-ATGCGGCGGGGGAAAAGGCG-3'; Human p38 reverse primer: 5'-GAACGACGCCGGAGGGCGCG-3'; Human ERK forward primer: 5'-GTCCAACCACAAGCTTTATC-3'; Human ERK reverse primer: 5'-CCATATTCCAACGCAGCGCA-3'.

Western Blotting

The total protein was extracted by radioimmunoprecipitation assay (RIPA) lysate. The concentration of each protein sample was determined by the bicinchoninic acid (BCA) kit. Briefly, 50 μg of total protein was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with 5% skimmed milk, followed by the incubation of specific primary antibodies (CD40, p-p38 MAKP, p-ERK) overnight. Membranes were then incubated with the secondary antibody at room temperature for 1 h. Immunoreactive bands were exposed by enhanced chemiluminescence method.

ELISA Assay

2 mL of peripheral blood from AS patients and normal controls were harvested for serum extraction. Serum expression of IL-10 was determined according to the instructions of ELISA kit.

Statistical Analysis

Statistical Product and Service Solutions (SPSS22.0, IBM Corp., Armonk, NY, USA) statistical software was used for data analysis. Measurement data were expressed as mean ± standard deviation ($\bar{x} \pm s$). Measurement data were compared using *t*-test. Classification data were compared using chi-square test. GraphPadPrism6.0 (GraphPad Software, La Jolla, CA, USA) was introduced for image editing. $p < 0.05$ was considered statistically significant.

Results

Changes of Lymphocyte Number and Surface Expression of CD40 in B Cells in AS Patients

PBMCs were extracted from peripheral blood samples of AS patients and normal controls. Numbers of T and B cells were detected by flow cytometry. Our data showed that no significant differences in numbers of T and B cells were found between AS patients and normal controls (Figure 1A). Furthermore, PBMCs were selected by flow cytometry (Figure 1B), and surface expression of CD40 in B cells was detected after CD19-positive cells (B cells) were circled out (Figure 1C). Our data indicated that no significant differences in surface expressions of CD40 in B cells were observed between AS patients and normal controls (Figure 1D and 1E). Moreover, no significant differences in protein expressions of CD40 in peripheral blood were found between AS patients and normal controls (Figure 1F).

Inhibited Activation of B Cells in Peripheral Blood of AS Patients

To examine whether B cells activation is abnormal in AS patients, CD40 expression was detected by flow cytometry after rCD154 treatment for 15 min and 30 min, respectively (Figure 2A and 2B). The results showed that surface expressions of CD40 in B cells were significantly increased in normal controls after being stimulated by rCD154 *in vitro*. However, no significant alteration of surface expressions of CD40 in B cells was observed in AS patients, indicating that B cells activation in AS patients was inhibited. Similar results were also obtained by Western blot (Figure 2C and 2D).

MAPK Pathway in Peripheral Blood B cells of AS Patients Was Inhibited

MAPK pathway-related molecules are downstream genes of CD40. Therefore, we speculated that abnormal expressed CD40 in AS patients would affect these molecules. We detected pho-

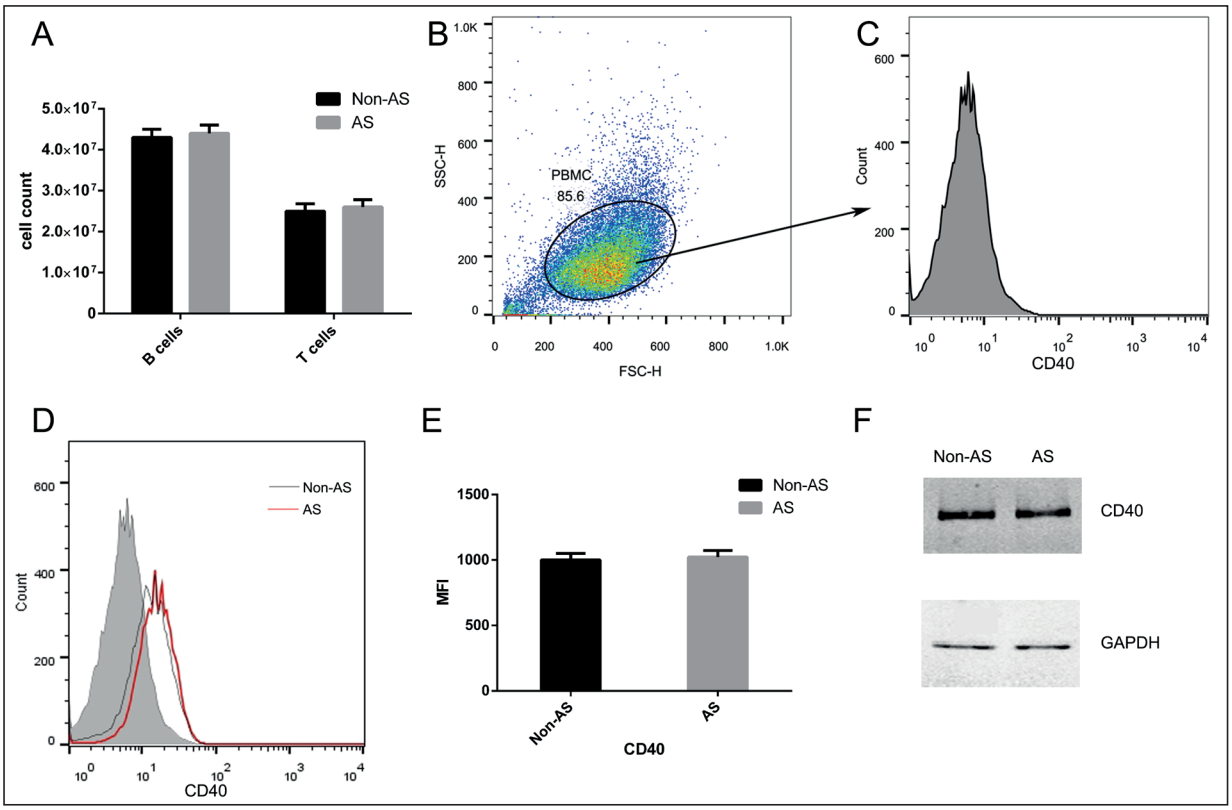

Figure 1. The number of T and B cells in AS patients and surface expression of CD40 in B cells. ***A,*** The number of T and B cells in AS patients and normal controls were detected by flow cytometry. ***B,*** and ***C,*** PBMCs (B) and CD19-positive cells (B cells) were screened out to detect surface expression of CD40 in B cells (C). ***D,*** Surface expression of CD40 in B cells of AS patients and normal controls were detected by flow cytometry. ***E,*** Surface expression of CD40 in B cells. ***F,*** Surface expressions of CD40 in B cells of AS patients and normal controls were detected by Western blot.

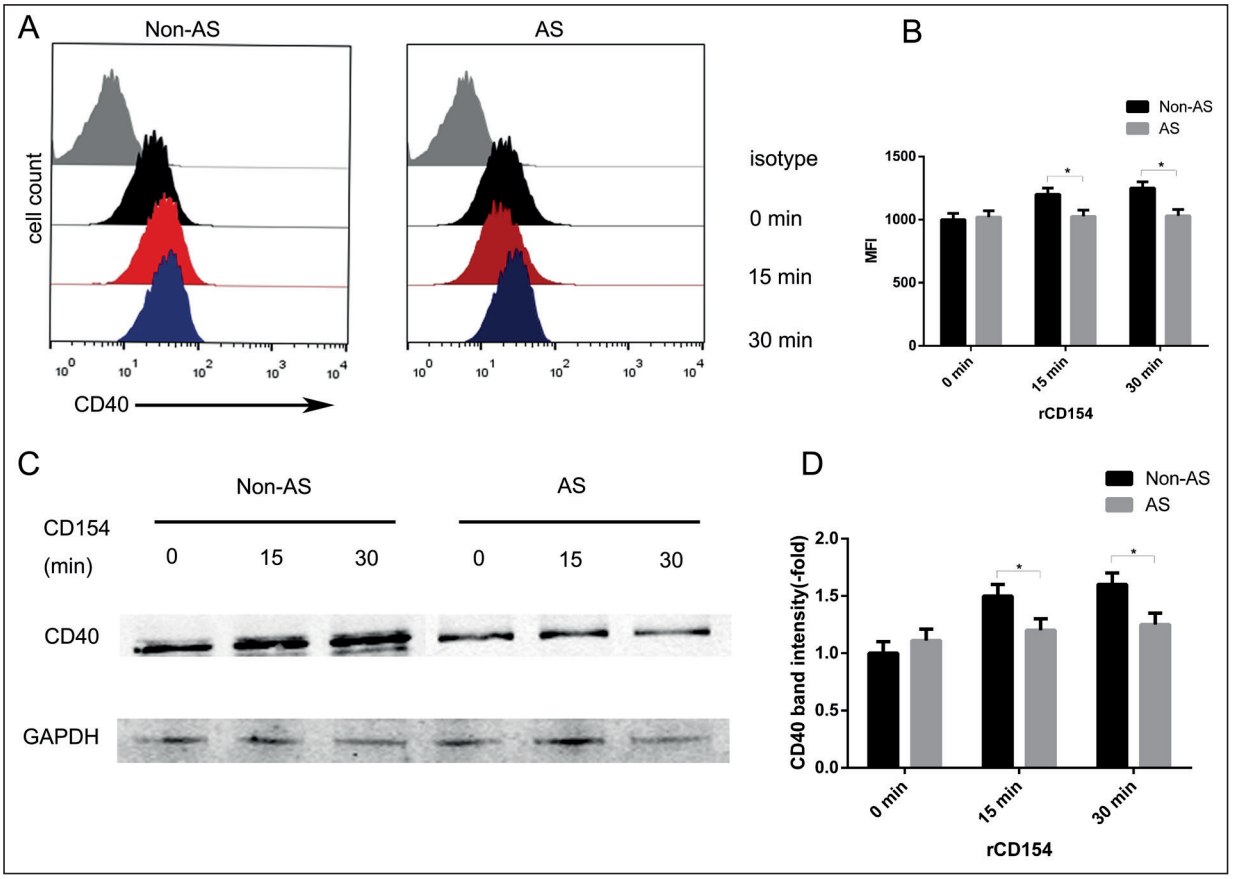

Figure 2. B cells activation was inhibited in AS patients. ***A,*** Surface expression of CD40 in B cells was detected by flow cytometry after rCD154 stimulation for 15 min and 30 min, respectively. ***B,*** Quantitative results of surface expression of CD40 in B cells. ***C,*** Surface expression of CD40 in B cells were detected by Western blot after rCD154 stimulation for 15 min and 30 min, respectively. ***D,*** Quantitative results of protein expression of CD40.

sphorylation levels of p38 MAPK (p-P38) and ERK (p-ERK) by Western blotting. After CD154 treatment, phosphorylation levels of p38 in B cells of AS patients were not significantly altered, which were significantly increased in normal controls (Figure 3A-C). However, no evident activation of ERK was observed in AS patients and normal controls (Figure 3A-C). We obtained similar results by qRT-PCR (Figure 3D and 3E).

IL-10 Secretion in Peripheral Blood Was Decreased in AS Patients

B cells are capable of differentiating into plasma cells and secreting various inflammatory factors. Abnormally activated B cells would affect the secretion of related inflammatory factors, thus interfering with the immune regulation in AS patients. Here, we measured the IL-10 amount in the peripheral blood of AS patients using ELISA. We found that there is a remarkable reduction in the IL-10 amount of AS patients (Figure 4A). Furthermore, PBMCs from peripheral blood of AS patients were isolated and cultured *in vitro*. After LPS stimulation for 24 h, PBMCs were harvested for further mRNA determination. The results demonstrated that mRNA levels of IL-10 in AS patients are remarkably decreased than those of the normal controls. However, the differences were not statistically significant (Figure 4B). Moreover, after B cells were treated with CD154 for 15 min, lower mRNA levels of IL-10 were observed in AS patients than those of normal controls ($p < 0.05$, Figure 4C).

Discussion

It is well recognized that B cells are involved in a number of immune regulators. B cell antigen receptor (BCR) is presented on the cell surface, which is capable of recognizing specific antigens[16]. When exposed to some antigens, B cells

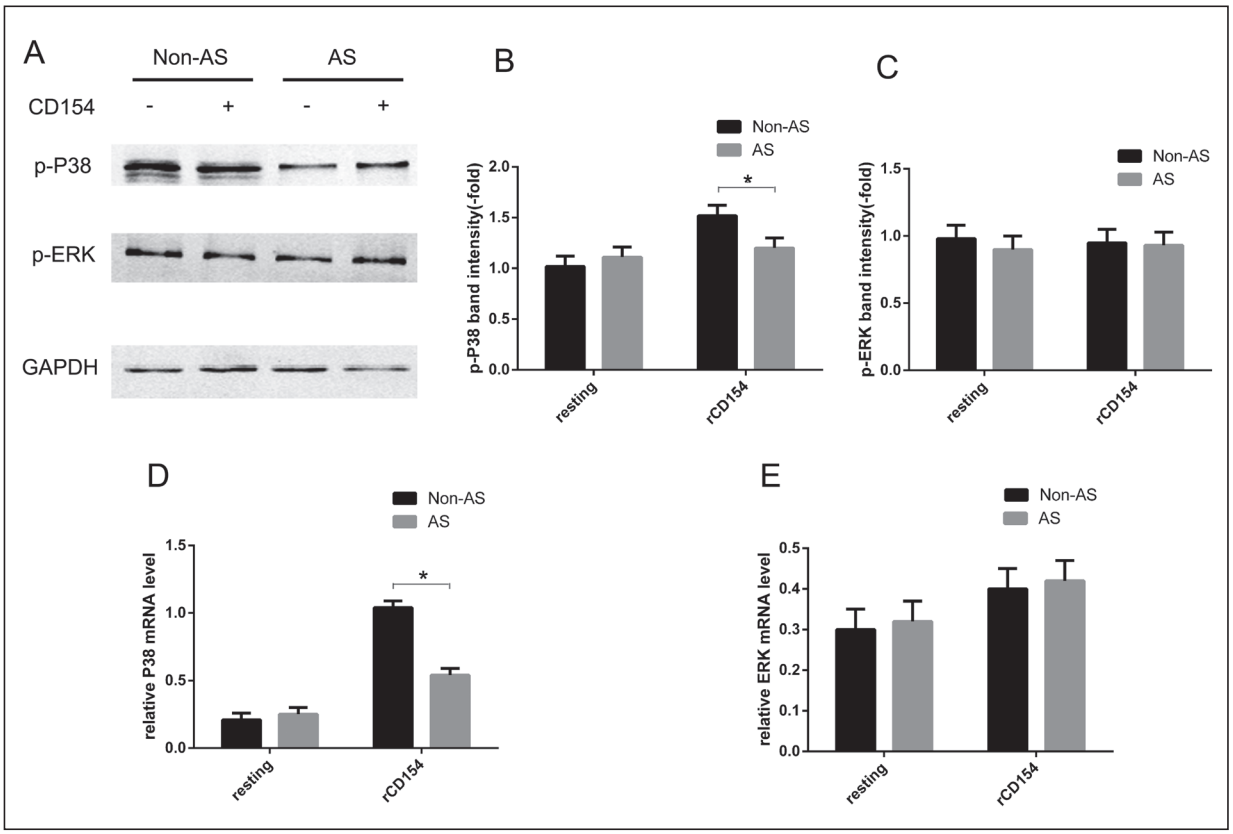

Figure 3. MAPK pathway was inhibited in peripheral blood B cells of AS patients. ***A,*** Levels of phosphorylated p38 and phosphorylated ERK in B cells were detected by Western blot after rCD154 treatment for 15 min. ***B,*** and ***C,*** Protein contents of phosphorylated p38 and phosphorylated ERK in B cells. ***D,*** and ***E,*** The mRNA levels of p38 (D) and ERK (E) in B cells were detected by RT-PCR after rCD154 treatment for 15 min.

would differentiate into plasma cells (effect or B cells), which in turn produce antibodies that are involved in immune regulation[17]. B cells not only regulate inflammation by secreting cytokines, such as IL-10, IL-8, and MCP-1[18], but also regulate immune response by interaction with T cells, macrophages, and dendritic cells[19]. Studies[20] have indicated that B cells are widely involved in many immunological diseases such as systemic lupus erythematosus, rheumatoid arthritis, scle-

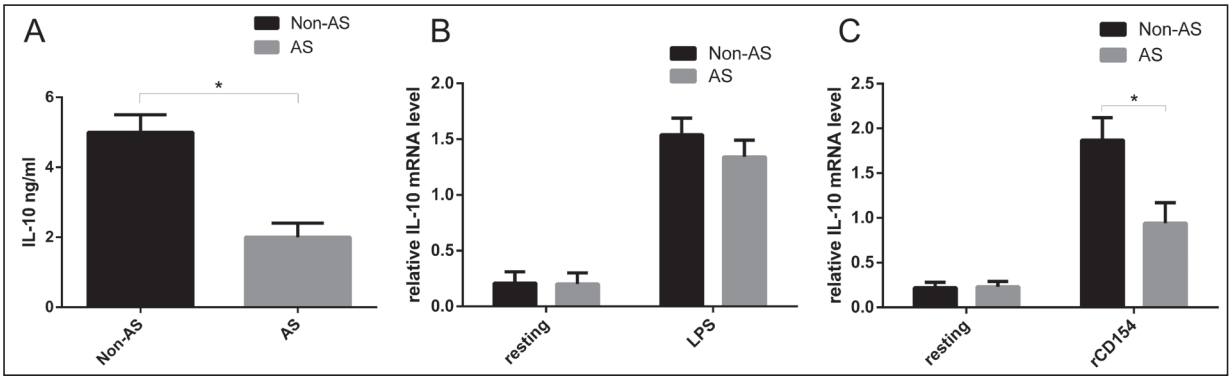

Figure 4. Decreased IL-10 secretion in peripheral blood of AS patients. ***A,*** Serum levels of IL-10 in AS patients and normal controls were detected by ELISA. ***B,*** Intracellular mRNA level of IL-10 was detected after LPS stimulation for 24 h. ***C,*** The mRNA level of IL-10 was detected after rCD154 treatment for 15 min.

roderma, and hyperthyroidism. Our investigation found that abnormal activated B cells in AS patients have essential clinical guidance for the AS immunotherapy.

CD40 is the type I transmembrane glycoprotein expressed on B cells surface, which is a member of the tumor necrosis factor receptor (TNF-α) superfamily. CD40 is the complementary glycoprotein of CD154 on T cells surface. CD40/CD154 interaction is responsible for regulating T cells activation[21]. CD40 has no catalytic activity; its intracellular domain, however, can recruit TNF-α. Subsequently, multimeric CD40 activates PI3K/Akt, p38 MAPK and NF-kB signaling pathways[22]. Additionally, CD40-CD40L interaction is involved in mediating inflammatory cytokines, such as MCP-1 and IL-10 *via* p38 MAPK pathway[23]. Our results indicated that after activation of B cells in peripheral blood of AS patients, surface expression of CD40 in B cells can't be normally increased. Therefore, the activation of its downstream signaling pathway was inhibited, which inevitably affected the normal immune function of B cells.

Mitogen-activated protein kinase (MAPK) is widely expressed in eukaryotes[24], which exerts an essential role in cell proliferation, apoptosis, and inflammation[25]. MAPK signaling pathways include the p38 protein kinase pathway, extracellular regulated protein kinase (ERK) pathway, and stress-activated protein kinase pathway (JNK)[26]. Abnormal activated p38 MAPK pathway was found in B cells of AS patients. However, we did not observe the abnormally activated ERK pathway, suggesting that abnormal expressed CD40 would not lead to complete inhibition of MAPK pathway. There are many downstream molecules in the MAPK pathway, including IL-10, IL-8, IL-6, and IL-12[27,28]. We found that IL-10 secretion in peripheral blood of AS patients are decreased, indicating that abnormally activated MAPK pathway can affect IL-10 secretion in AS patients.

We also detected that no significant changes in surface expressions of CD40 in B cells of AS patients were found compared with those of normal controls. However, abnormal surface expression of CD40 in B cells inhibited the activation of p38 MAPK pathway, resulting in a decreased secretion of the IL-10 and an abnormal activation of the B cells. Therefore, the abnormally activated B cells in peripheral blood of AS patients are involved in the development and progression of AS, which have important clinical guidance for the AS immunotherapy.

Conclusions

We showed that the abnormal surface expressions of CD40 in B cells of AS patients lead to abnormal activation of B cells, which downregulate the IL-10 secretion *via* inhibition of the p38 MAPK pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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