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Correlations of expressions of Toll-like receptor 4 and Janus kinase 3 in peripheral blood mononuclear cells with T helper 17 cell/ regulatory T cell imbalance in patients with ankylosing spondylitis

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ABSTRACT

Background: We aimed to analyze the correlations of the expressions of Toll-like receptor 4 (TLR4) and Janus kinase 3 (JAK3) in peripheral blood mononuclear cells (PBMCs) with T helper 17 cell (Th17)/ regulatory T cell (Treg) imbalance in patients with ankylosing spondylitis (AS).

Methods: A total of 101 AS patients were enrolled into an AS group. Based on the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) score, they were subdivided into a stable AS (ASS) group (BASDAI score <4 points, n=42) and an active AS (ASA) group (BASDAI score ≥4 points, n=59). Meanwhile, 50 healthy volunteers who received health examination in the same time period were assigned into a healthy control (HC) group. RT-qPCR was performed to determine the mRNA expressions of TLR4 and JAK3 in PBMCs. Enzyme-linked immunosorbent assay was conducted to measure the levels of Th17/ Treg-related cytokines [interleukin (IL)-10, IL-17, IL-23, and transforming growth factor-β (TGF-β)] in the serum.

Results: The mRNA expressions of TLR4 and JAK3 had positive correlations with IL-17 (r=0.592, 0.542) and IL-23 levels (r=0.529, 0.590) (P 0.05). The levels of TLR4 mRNA, JAK3 mRNA, IL-17, and IL-23 were all correlated with the occurrence of AS (P<0.05). The areas under the receiver operating characteristic curves of TLR4, JAK3, and their combination in PBMCs for predicting the occurrence of AS were 0.912, 0.835, and 0.919, respectively.

Conclusions: TLR4 combined with JAK3 can effectively predict the occurrence of AS.

Keywords: ankylosing spondylitis, correlation, expression, mononuclear cell, peripheral blood

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INTRODUCTION

As a chronic autoimmune inflammatory disease mainly characterized by axial joint involvement and affecting the spinal osteoid process, sacroiliac joint, and paraspinal soft tissue, ankylosing spondylitis (AS) can cause spine and joint stiffness as well as peripheral arthropathy [1, 2]. Frequently occurring in young and middle-aged males, AS can be classified into stable AS (ASS) and active AS (ASA) based on the activity. Currently, the pathogenesis of AS has not been fully elucidated yet, which is generally considered related to such factors as genetics, environment, immunological abnormality, and inflammatory response, among which immuno-inflammatory response plays an important role [3, 4]. The Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathway is a crucial intracellular signal transduction pathway during immunoregulation, and plenty of cytokines can mediate the inflammatory response by regulating this pathway [5]. A recent study reported that abnormal JAK3 gene expression is related to various rheumatic immune diseases, and JAK inhibitors are fairly feasible and safe in treating AS [6]. Toll-like receptor 4 (TLR4), a type I transmembrane protein, serves as a bridge between adaptive immunity and innate immunity and recognizes the pathogen-associated molecular patterns of different pathogens, thus inducing the expressions of pro-inflammatory and immune-related genes and further exacerbating the inflammatory response [7]. Additionally, TLR4 is expressed on the cell membrane surface and in synovial cells, and can cause sustained inflammatory cell activation by activating the NF-κB signaling pathway, thus aggravating synovial inflammation and accelerating the occurrence of diseases such as osteoarthritis [8]. To further uncover the pathogenesis of AS, the mRNA expressions of TLR4 and JAK3 in peripheral blood mononuclear cells (PBMCs) of AS patients were observed, and their correlations with helper T 17 cell (Th17)/regulatory T cell (Treg) imbalance were analyzed in this study.

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METHODS

General data

This study has been approved by the ethics committee of our hospital on January 4th, 2022 (No. XCH202201003). A total of 101 AS patients diagnosed in our hospital from January to December 2022 were enrolled into an AS group, including 77 males and 24 females aged 18-75 years old [(40.49±4.85) years old on average], with a mean body mass index (BMI) of (23.15±1.05) kg/m2. There were 42 cases of ASS and 59 cases of ASA. In addition, 50 healthy volunteers (without previous history of AS and family history of AS) who received health examination in the same period were assigned to a healthy control (HC) group. There were 35 males and 15 females with the age of 20-75 years, (41.03±3.89) years on average. The mean BMI was (22.85±1.12) kg/m2. The gender, age, and BMI were comparable between the AS and HC groups (P>0.05).

Inclusion and exclusion criteria

The inclusion criteria were set as follows: 1) patients meeting the diagnostic criteria of AS and definitely diagnosed by imaging examinations, accompanied by reduced thoracic mobility [9], limited lumbar mobility, morning stiffness, and low back pain, 2) those who were diagnosed for the first time, 3) those aged 18-75 years of age, 4) those who did not receive immunomodulatory and glucocorticoid treatments within 3 months before enrollment, and 5) those who voluntarily signed the informed consent.

The exclusion criteria involved 1) patients also suffering from other connective tissue diseases, infectious diseases, or hematological diseases, 2) those with severe vital organ (heart, brain, liver, or kidney) dysfunction, 3) those with other types of rheumatic diseases, 4) those who had a history of immune-related diseases, viral hepatitis, or other types of autoimmune diseases, or 5) pregnant or lactating women.

Grouping method for AS group

The Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) score was utilized to evaluate the disease activity of patients after admission [10], where the patients were instructed to answer a total of 6 questions based on symptoms over the past week, including A) the overall degree of fatigue/drowsiness (0-10 points), B) the overall degree of neck pain, back pain, and hip pain (0-10 points), C) the overall degree of pain or swelling in other joints (0-10 points), D) the degree of discomfort at the site of tender pain or pressing pain (0-10 points), E) the degree of stiffness when waking up (0-10 points), and F) the duration of joint stiffness calculated from waking up (0, 30, 60, 90 and more than 120 min valued as 0, 2.5, 5, 7.5 and 10 points, respectively). The total score was calculated based on the formula: total score = 0.2 × [A + B + B + D + (E + F)/2], ranging from 0 to 10 points. Patients with BASDAI score <4 points were allocated into an ASS group, while those with BASDAI score ≥4 points were included in an ASA group.

Detection of TLR4 and JAK3 mRNA expressions in PBMCs

Fasting venous blood (3 mL) was collected from the AS group before treatment and from the HC group on the day of health examination into heparin anticoagulant tubes (Hepeng [Shanghai] Biological Technology Co., Ltd., China; catalog number: HPBIO-JC61), from which PBMCs were isolated and purified by Ficoll-Paque PLUS kit (GE Healthcare Life Sciences, USA; catalog number: 17-1140-02). Besides, the total number of PBMCs was determined using Countess II Automated Cell Counter (Thermo Scientific, USA; catalog number: AMQAX1000), and the cells were diluted with PBS (Beijing Bossbio Co., Ltd., China; catalog number: IH0001/500) to the density of 2×109 cells/L. Next, a portion of cell suspension (100 μL) was processed to extract total RNA through TRIzol RNA extraction kit (BioTeke Corporation (Wuxi) Co., Ltd., China; catalog number: RP2402). Then it was purified and reverse-transcribed into cDNA using a reverse transcription kit (Jiangsu Maige Biological Technology Co., Ltd., China; catalog number: K16225-100T) with a 20 μl reaction system. Afterwards, an equal amount of the reverse transcription product was obtained to measure the mRNA expressions of TLR4 and JAK3 by quantitative polymerase chain reaction (qPCR) using MiniAmp Thermal Cycler (Thermo Scientific, USA; catalog number: A37834). The reaction conditions were: (94°C for 50 min, 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s) × 30 cycles, 72°C for 5 min, and 4°C for 5 min. The primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd. (China), including TLR4-forward (F): 5'-ACTTGGACCTTTCCAGCAAC-3', TLR4-reverse (R): 5'-TTTAAATGCACCTGGTTGGA-3', JAK3-F: 5'-CTCCTTCCGAGCCGTCAT-3', JAK3-R: 5'-CTCCCGCTGAAAGTCCCT-3', β-actin-F: 5'-GTGGGGCGCCCCAGGCACCA-3', and β-actin-R: 5'-CTCCTTAATGTCACGCACGATTTC-3'. The relative mRNA expressions of TLR4 and JAK3 were determined using the 2-ΔΔCt method. ΔΔCt = (Ct, target - Ct, β-actin) AS group- (Ct, target- Ct, β-actin) HC group.

Detection of serum Th17/ Treg-related cytokines

Fasting venous blood (3 mL) was collected from the AS and HC groups before treatment and on the day of health examination into plain tubes (Henan Medical Device Co.,

Ltd., China; catalog number: 211011), which was centrifuged at 3,000 r/min for 15 min (with a centrifugal radius of 10 cm) to separate the serum. Subsequently, the Th17/Treg-related cytokines in the serum were determined by means of enzyme-linked immunosorbent assay. The kit for interleukin (IL)-10 was purchased from Wuhan Yipu Biotechnology Co., Ltd. (China; catalog number: CSB-E04593h-IS; detection limit: 3.9-250 pg/ml). The kits for IL-17 and IL-23 were bought from Wuhan Fein Biotechnology Co., Ltd. (China; catalog numbers: EH2513 and H3270, respectively; detection limits: 15.625-1000 pg/ml and 39.063-2500 pg/ml, respectively). The kit for transforming growth factor-β (TGF-β) was obtained from Chuzhou Sinoda Biotechnology Co., Ltd. (China; catalog number: SND-H1905; detection limit: 6.25-400 pg/ml).

Statistical analysis

EpiData software (EpiData Association, Denmark) was applied for data entry, and SPSS 23.0 software (IBM Inc., USA) was used for statistical analysis. All measurement data were tested for normality. The normally distributed measurement data (TLR4 mRNA, JAK3 mRNA, Th17/Treg-related cytokines) were expressed as mean ± standard deviation (`x ± s). In the case of homogeneity of variance, the independent-samples t-test was used for intergroup comparisons, and the paired samples t-test was utilized for intragroup comparisons. The corrected t-test (t'-test) was performed in the case of heterogeneity of variance. The non-normally distributed measurement data were described by median and interquartile range, and subjected to the Mann-Whitney U test. The categorical data were presented as percentages and examined by the $\chi 2$ test. Correlations were explored using Pearson analysis. Linear regression analysis was conducted to investigate the influencing factors for the occurrence of AS. Receiver operating characteristic (ROC) curves were plotted to analyze the predictive value. P<0.05 suggested that the difference was statistically significant.

RESULTS

TLR4 and JAK3 mRNA expressions in PBMCs in AS and HC groups

The mRNA expressions of TLR4 and JAK3 in PBMCs were higher in the AS group than those in the HC group (P<0.05) (Table 1).

Levels of Th17/ Treg-related cytokines in the peripheral blood of AS and HC groups

The AS group had increased levels of IL-17 and IL-23 in the peripheral blood compared with those in the HC group (P<0.05), but the IL-10 and TGF-β levels in the peripheral blood were not significantly different between the AS group and the HC group (P>0.05) (Table 2).

Correlations of mRNA expressions of TLR4 and JAK3 in the peripheral blood with Th17/ Treg-related cytokines

According to Pearson correlation coefficients, the mRNA expressions of TLR4 and JAK3 had positive correlations with IL-17 (r=0.592, 0.542) and IL-23 levels (r=0.529, 0.590) (P<0.05) but no significant correlations with IL-10 (r=-0.105,-0.107) or TGF-β level (r=-0.120,-0.098) (P>0.05). Besides, TLR4 mRNA expression was positively correlated with JAK3 mRNA expression (r=0.615, P<0.05) (Table 3).

Results of linear regression analysis

It was found through linear regression analysis that the levels of TLR4 mRNA, JAK3 mRNA, IL-17, and IL-23 were all correlated with the occurrence of AS (P<0.05), whereas no correlation between the levels of IL-10 and TGF-β and the occurrence of AS was observed (P>0.05) (Table 4).

Table 1. TLR4 and JAK3 mRNA expressions in PBMC from AS and HC groups

Group	n	TLR4 mRNA	JAK3 mRNA
HC	50	1.57 ± 0.53	1.34 ± 0.51
AS	101	2.69 ± 0.71	1.99 ± 0.51
t value		9.954	7.482
P value		< 0.001	< 0.001

The relative mRNA expressions of TLR4 and JAK3 (mean ± SD) were determined by qPCR using the 2-ΔΔCt method. Abbreviations: AS- ankylosing spondylitis, HC- healthy control, JAK3- Janus kinase 3, PBMC- peripheral blood mononuclear cell, SD- standard deviation, TLR4- toll-like receptor 4.

Table 2. Levels of Th17/ Treg-related cytokines in the peripheral blood of AS and HC groups

Group	n	Th17-related cytokine		Treg-related cytokine	
		IL-17	IL-23	IL-10	TGF-β
HC	50	8.25 ± 1.95	2332.60 ± 505.87	193.38 ± 36.24	417.97 ± 55.84
AS	101	11.69 ± 2.52	3123.90 ± 625.15	192.69 ± 32.25	429.87 ± 44.55
t value		8.463	7.775	0.119	1.418
P value		< 0.001	< 0.001	0.906	0.158

The concentrations were detected by enzyme-linked immunosorbent assay and are expressed as mean ± SD, in ng/L. Abbreviations: AS- ankylosing spondylitis, HC- healthy control, IL- interleukin, SD- standard deviation, TGF-β- transforming growth factor-β, Th17- T helper 17 cell, Treg- regulatory T cell.

Table 3. Peripheral TLR4 and JAK3 mRNA expression correlation with Th17/ Treg-related cytokines

Index	Coefficient	TLR4 mRNA	JAK3 mRNA	IL-17	IL-23	IL-10	TGF-β
TLR4 mRNA	r value	-	0.615	0.592	0.529	-0.105	-0.120
	P value	-	< 0.001	< 0.001	0.001	0.198	0.141
JAK3 mRNA	r value	0.615	-	0.542	0.590	-0.107	-0.098
	P value	< 0.001	-	< 0.001	< 0.001	0.192	0.232

Correlations were explored using Pearson analysis. Abbreviations: IL- interleukin, JAK3- Janus kinase 3, TGF-β- transforming growth factor-β, Th17- T helper 17 cell, TLR4- toll-like receptor 4, Treg- regulatory T cell.

Table 4. Results of linear regression analysis

Item	β	Standard error	Standard coefficient	t value	P value	95% confidence interval
Constant	-0.635	0.128	-	-4.971	< 0.001	-0.887 to 0.382
TLR4 mRNA	0.218	0.043	0.389	5.051	< 0.001	0.133- 0.303
JAK3 mRNA	0.415	0.055	0.523	7.482	< 0.001	0.306- 0.525
IL-17	0.037	0.013	0.226	2.884	0.005	0.012- 0.063
IL-23	0.041	0.018	0.238	3.002	0.003	0.015- 0.079
IL-10	0.000	0.001	-0.023	-0.277	0.782	-0.003 to 0.002
TGF-β	0.001	0.001	0.118	1.435	0.153	0.000- 0.003

Abbreviations: IL- interleukin, JAK3- Janus kinase 3, TGF-β- transforming growth factor-β, TLR4- toll-like receptor 4.

Table 5. Predictive values of PBMC TLR4 and JAK3 mRNA expressions for the occurrence of AS

Index	Optimal cut-off value	AUC	Standard error	95% CI	P value	Sensitivity	Specificity
TLR4 mRNA	2.085	0.912	0.028	0.866-0.959	< 0.001	0.852	0.805
JAK3 mRNA	1.641	0.835	0.039	0.759-0.911	< 0.001	0.764	0.811
TLR4 + JAK3	-	0.919	0.024	0.867-0.973	< 0.001	0.861	0.808

Abbreviations: AS- ankylosing spondylitis, AUC- area under the ROC curve, CI- confidence interval, JAK3- Janus kinase 3, PBMC- peripheral blood mononuclear cell, TLR4- toll-like receptor 4.

Table 6. Levels of TLR4, JAK3 and Th17/ Treg-related cytokines in ASA and ASS groups

	n	TLR4 mRNA	JAK3 mRNA	Th17-related cytokines		Treg-related cytokines	
				IL-17	IL-23	IL-10	TGF-β
ASS group	42	2.02 ± 0.35	1.51 ± 0.25	9.84 ± 1.35	2643.67 ± 426.17	200.84 ± 31.75	445.11 ± 38.12
ASA group	59	3.17 ± 0.47	2.34 ± 0.33	13.00 ± 2.33	3465.75 ± 509.46	186.88 ± 31.60	419.02 ± 45.90
t value		13.410	13.668	7.902	8.541	2.185	3.015
P value		< 0.001	< 0.001	< 0.001	< 0.001	0.031	0.003

All results (relative expression for mRNA and ng/L for cytokines) are shown as mean ± SD. Abbreviations: ASA- active ankylosing spondylitis, ASS- stable ankylosing spondylitis, IL- interleukin, JAK3- Janus kinase 3, SD- standard deviation, TGF-β- transforming growth factor-β, Th17- T helper 17 cell, TLR4- toll-like receptor 4, Treg- regulatory T cell.

Table 7. Correlations of TLR4, JAK3 and Th17/ Treg-related cytokines with BASDAI score of AS patients

	TLR4	JAK3	IL-17	IL-23	IL-10	TGF-β
r value	0.615	0.632	0.502	0.532	-0.385	-0.391
P value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

Abbreviations: AS- ankylosing spondylitis, BASDAI- Bath Ankylosing Spondylitis Disease Activity Index, IL- interleukin, JAK3- Janus kinase 3, TGF-β- transforming growth factor-β, Th17- T helper 17 cell, TLR4 - toll-like receptor 4, Treg- regulatory T cell.

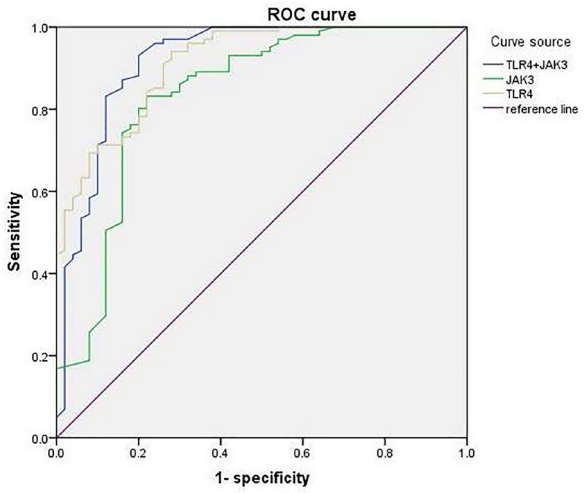

Figure 1. Receiver operating characteristic curves of mRNA expressions of toll-like receptor 4 (TLR4) and Janus kinase 3 (JAK3) in peripheral blood mononuclear cells (PBMC) for predicting the occurrence of ankylosing spondylitis (AS).

Predictive values of TLR4 and JAK3 mRNA expressions in PBMCs for occurrence of AS

With the presence or absence of AS (0 for absence and 1 for presence) as a state variable and the expressions of TLR4 and JAK3 in PBMCs as the test variables, the analysis results of the ROC curves indicated that the area under the ROC curve (AUC) of TLR4, JAK3 and their combination in PBMCs for predicting the occurrence of AS was 0.912 (95% CI: 0.866-0.959), 0.835 (95% CI: 0.759-0.911), and 0.919 (95% CI: 0.867-0.973), respectively (Table 5 and Figure 1).

Levels of TLR4, JAK3, and Th17/ Treg-related cytokines in ASA and ASS groups

The ASA group presented raised mRNA expressions of TLR4 and JAK3 in PBMCs, increased IL-17 and IL-23 levels in the serum, and decreased IL-10 and TGF-β levels in the peripheral blood in comparison with the ASS group (P<0.05) (Table 6).

TLR4, JAK3, and Th17/ Treg-related cytokines correlation with the BASDAI score of AS patients

Pearson correlation coefficients revealed positive correlations of BASDAI score with TLR4, JAK3, IL-17, and IL-23 (r=0.615, 0.632, 0.502, 0.532) (P<0.05), as well as a negative correlation between IL-10 and TGF-β and BASDAI score (r=-0.385,-0.391) (P<0.05) (Table 7).

DISCUSSION

AS has insidious onset and irregular changes in its condition, and ASS and ASA usually emerge alternately. ASA is an acute manifestation of aggravated inflammatory responses and relapse, which can increase the degrees of motion limitation and tissue injury and further reduce spinal mobility [11, 12]. Moreover, AS can elevate the risk of spinal deformity with the disease progression, thus seriously affecting patients' quality of life. Therefore, distinguishing AS early and identifying the spinal mobility are particularly crucial for early detection, early diagnosis and early treatment.

As a member of the membrane protein family, TLR4 mediates NF-κB activation and activates intracellular signaling pathways in vivo through the MyD88-dependent and -independent signaling pathways after recognizing its ligands, resulting in the massive synthesis and release of many inflammatory factors and adhesion factors, and thus exacerbating inflammatory response and immune cell activation [13, 14]. The JAK/STAT3 signaling pathway can be triggered by pro-inflammatory cytokines, which are extensively implicated in cell apoptosis and growth, oxidative stress, inflammation, and other processes. The JAK family consists of JAK1, JAK2, and JAK3. Among them, JAK3 is highly expressed in hematopoietic tissues, and its deletion may lead to disorders in pro-inflammatory and anti-inflammatory functions involving lymphocytes, thereby influencing immune function [15]. Liao et al. employed real-time RT-qPCR assay to determine the mRNA expression of JAK1 and JAK3 in PBMCs of 31 HCs and AS patients [16]. They found that JAK1 and JAK3 exhibited high mRNA expressions in PBMCs of AS patients, and their levels were positively correlated with C-reactive protein, erythrocyte sedimentation rate, and human leukocyte antigen B27 in the serum, as well as uveitis and peripheral arthritis of the patients. Furthermore, the plotted ROC curves showed that JAK-3 mRNA effectively predicted the disease activity of patients. In this study, the AS group displayed raised mRNA expressions of TLR4 and JAK3 in PBMCs in contrast with the HC group, and linear regression analysis revealed that the high expressions of TLR4 and JAK3 were correlated with the incidence of AS. The possible reasons are as follows. First, TLR4 can attenuate the barrier function of epithelial cells by up-regulating the expressions of autoreceptors and certain chemokines, thus causing neutrophil migration and inducing inflammatory responses [17]. Meanwhile, TLR4 can also activate a series of immunocompetent cells such as endothelial cells, macrophages, and lymphocytes, promote the maturation and activation of cytotoxic T cells, and inhibit the immune function in organisms [18]. Second, JAK3 overexpression can act on lymphocytes, increase the secretion of pro-inflammatory factors including IL-6 and IL-21, and exacerbate inflammatory responses, thereby causing cartilage destruction and synovial proliferation and triggering AS [19].

Th17 and Treg are in a balanced state in healthy people, and they can achieve cell transformation via some cytokines. When the in vivo cellular environment changes, however, the Th17/Treg imbalance occurs, further promoting the progression of autoimmune diseases [20, 21]. Liao and Tsai verified the correlations among T-regs, cytokines, disease activity scores, and inflammatory reactants in AS patients [22]. Liu et al. incorporated 95 pieces of literature (3,065 HCs and 4,020 AS patients) into a meta-analysis, manifesting an increased proportion of Th17 and a decreased proportion of Treg in the peripheral blood of AS patients compared to those of HCs, as well as prominent Th17/Treg imbalance in AS patients [23]. Moreover, Fattahi et al. reported that AS patients had higher levels of serum IL-17, IL-16, TNF-α, and Th17 than normal controls, but there were no significant differences in IL-10 and Treg levels between the two groups, suggesting that targeting Th17 or cytokines secreted by Th17 alone can also correct cytokine imbalance [24]. It was discovered through the present study that the levels of IL-17 and IL-23 in the peripheral blood were higher in the AS group than those in the HC group, and the ASA group presented elevated levels of serum IL-17 and IL-23 in comparison to the ASS group, but the levels of IL-10 and TGF-β were not remarkably different between the AS and HC groups, which are similar to the research results of Fattahi et al. [24], further validating potential Th17 and Treg disorders in AS patients. Furthermore, Pearson correlation analysis in this study uncovered that the mRNA expressions of TLR4 and JAK3 were positively correlated with Th17/Treg-related cytokines (IL-17 and IL-23) and BASDAI score. Therefore, such mRNA expressions may be associated with the changes in Th17/Treg and disease activity in AS patients.

Nevertheless, this study is limited. The Th1/Treg imbalance is assessed indirectly by measuring the production of related cytokines. Besides, whether the lymphocyte imbalance can be modulated by the available drugs is not tested. Further studies are ongoing in our group to remedy the deficiency.

CONCLUSION

In conclusion, AS patients have high mRNA expressions of TLR4 and JAK3 in PBMCs and the Th17/Treg imbalance, TLR4 and JAK3 expressions are positively correlated with the Th17/Treg imbalance and disease activity. TLR4 combined with JAK3 may apply to the prediction of AS occurrence.

AUTHORS' CONTRIBUTION

RH- study design, data collection

GL and JY- data analysis.

FG- data collection

ML- writing the manuscript

All authors have read the complete manuscript and take responsibility for the content of the manuscript.

CONFLICT OF INTEREST

None to declare.

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