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Original Article

Detection of Toll-like receptors (TLR2, TLR4) expression and apoptosis of lymphocytes in patients with ankylosing spondylitis

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Abstract: The expression levels of the peripheral blood mononuclear cells surface Toll-like receptors (TLR2, TLR4), the quantity of B lymphocyte subgroups, and the number of apoptosis lymphocyte in peripheral blood were detected to investigate their implication in the occurrence and development of ankylosing spondylitis and propose a new system for monitoring patients and therapeutic strategies. We analyzed the peripheral blood specimens stemming from 67 Ankylosing spondylitis (AS) patients and 30 healthy individuals through flow cytometry. Two reagents, FITC TLR2 and PE-TLR4, were used for the detection of expression levels of TLR2 and TLR4 while APC-CD19 and PE-CD5 were used in the enumeration of different B lymphocytes subgroups. For the detection of lymphocytes apoptosis, we employed FITC and streaming Annexin-v/PI method. The expression levels of peripheral blood mononuclear cells surface TLR2 and TLR4 appeared unusually high in the patients with AS and was not associated with disease activity. Similarly, there were abnormal changes in the amount of B lymphocytes subset in the patients with AS. The amount of apoptosis lymphocytes in peripheral blood were reduced in AS patients which was correlated with the abnormal increase of CD19+ and CD19+CD5+ B lymphocytes. Apoptosis Lymphocytes as well as the amount of CD19+ and CD19+CD5+ B lymphocytes were all associated with AS disease activity. The present study provided new insights into the physiopathology and the pathogenesis of AS disease and suggested new strategies for the monitoring and treatment of AS patients.

Keywords: Ankylosing spondylitis, TLR2, TLR4, apoptosis, CD19+CD5+, CD19+CD5-, CD19+

Introduction

Ankylosing spondylitis (AS) is a *HLA-B27* as well as manifold minor genetic and environmental factors associated chronic and hereditary inflammatory rheumatic sickness which is encountered in a feeble proportion of Chinese and European populations. Besides, it mainly affects the vertebral column and pelvis and causes pain and stiffness to progressively leads to new bone formation and ankyloses of touched junctions [1, 2]. Meta-analyses have proved that the role of *TNF*-alpha promoter polymorphisms at positions -238, -308, -850, -857, -863 and -1031 in ankylosing spondylitis susceptibility [2]. Apart from the commonly known *HLA-B*27* alleles, a case-control study based on the Illumina Immunochip microarray led to the identification of immune-related loci associated with AS [1]. Scholars have equally shown the association of *RUNX3, LTBR-TNFRSF1A* and *IL12B* variants regions and loci at *PTGER4, TBKBP1, ANTXR2* and *CARD9* with AS disease and provided strong evidence that *HLA-B27* operates in AS through a mechanism involving aberrant processing of antigenic peptides by interacting with *ERAP1* which encodes an endoplasmic reticulum aminopeptidase enmeshed in peptide excision before *HLA* class I presentation [3]. Two loci, between *EDIL3* and *HAPLN1* at 5q14.3 and within *ANO6* at 12q12, implicating genes related to bone formation and cartilage development and belonging to the major histocompatibility complex (MHC) region (top SNP, rs13202464) and at 2p15 (rs10865331) are potentially involved in the etiology of AS [4]. AS was also found to be related to the gene involved in osteoclastogenesis, and

in the interaction between T cells and dendritic cells (SNP rs8092336) and the PTGS1 (prostaglandin-endoperoxide synthase 1, cyclooxygenase 1) gene (SNP rs1236913) [5]. The anthrax toxin receptor 2 (*ANTXR2*) gene (SNP rs12504282), independently from the HLA-*B27* variants, was reported to be plainly linkedto AS but its mechanism of action is not yet established [6]. In addiotion, the association with low vitamin D concentrations and higher AS disease activity was also investigated [7]. Research works have proposed bacterial and virus infections and genetic and immune factors as important causative elements of the disease while other scholars have classified AS as an autoimmune illness [8, 9].

Despite the broad knowledge about the etiology of AS, the pathogenesis at the immunological level is not fully scrutinized. The mechanisms that initiate bony fusion in AS need to be completely well-defined. In recent years, there have been several progresses in our interpretation of the pathogenesis of this multifaceted disease. Previous studies have shown the considerable increase of circulating *IL-17*-secreting γ/δ T cells, induced by the expression of the maturation and growth factor *IL-23* and/or anti-CD3/CD28, in patients with active AS and have conveyed the implication of these cells in AS pathogenesis [10, 11]. The increase of expressing the killer-cell Ig-like receptor KIR3DL2+ in Th17 cells and responsive to HLA-B27 homodimers in the peripheral blood of AS patients was previously reported and interconnected HLA-B27 with IL-17 production [12]. Moreover, elevated concentrations of Th22 cells and Th17 cells in the peripheral blood of AS patients have been exposed and incriminated in AS pathogenesis [13]. It was demonstrated that C-reactive protein (CRP) levels, frequently used to control disease activity in AS patients, fluctuate with different CRP genotype in AS patients and high levels of CRP do not necessarily correlate with high AS activity [14]. The probable contribution of higher levels of noggin (NOG) and/or sclerostin (SOST)-IgG immune complexes to the neo-ossification in AS patients was correspondingly published [15]. The prospective implication of levels of Wnt pathway regulatory proteins in T cell activation and differentiation and in bone marrow adipogenesis leading to the physiopathology and bony fusion in AS was also suggested [16]. Research based on Nucleic Acid Programmable Protein Arrays (NAPPA) showed multi-specific targeting connective tissue and skeletal proteins autoantibody responses to several autoantigens in AS patients and demonstrated the autoimmune manifestations of AS [17]. Similarly, anti-PPM1A (Protein Phosphatase Magnesium-Dependent 1A) autoantibodies were found in AS, suggesting the probable contribution of PPM1A to the pathogenesis of AS [18]. Studies have also reported the importance of innate immune response and the acquired immune response in the pathogenesis of AS. Whole genome expression profiling and signal pathway screening showed that AS pathogenesis is possibly due to the stimulation of the MAPK and Toll-Like Receptors (TLRs), signaling pathways [19].

In recent years, each subgroup of B lymphocytes has also been seriously surveyed and is widely spread in the phenomenon of immune activation and autoimmune diseases. Studies have shown abnormal occurrence of cellular and humoral immunity in AS patients.

The dynamic balance of cell proliferation and apoptosis is one of the important factors in maintaining the body's immune stability. The destruction of this balance may lead to the occurrence of a variety of autoimmune diseases. Literature reported that TLRs were narrowly connected to the apoptosis of neutrophils. Among TLRs, TLR2 plays a key role in the induction of apoptosis and anti-apoptotic effect. The increase of lymphocytes numbers and (or) apoptosis is associated with the occurrence of the disease development in AS patients. CD19+CD5+ B lymphocytes are long-living cells capable of self-renewal functions that can abnormally express Fas protein molecules in SLE patients and cause abnormal changes of apoptosis mechanism. However, the correlation between TLR2, changes in B lymphocytes subset and lymphocytes apoptosis and the occurrence of the disease in AS patients are not reported so far.

Therefore, we detected the peripheral blood mononuclear cells surface TLR2 and TLR4, the enumeration of B lymphocyte subsets and the level of lymphocyte apoptosis in AS patientsto study their impact and their correlation with the occurrence and development of AS, which illuminated new insights into the symptoms of the disease and providing new ideas for clinical diagnosis, patient monitoring and treatment methods.

Material and methods

Experimental groups

We collected peripheral blood samples from 67 HLA-B27 positive AS patients from June 2013 to November 2013 at the Affiliated Hospital of Guiyang Medical Colleges. We adopted the 1984's New York criteria as amended inclusion criteria [14]. The studied population included 53 males and 14 females with age comprised between 9-58 and an average age of 28. The average age of men was 27 years old while the average age of women was 30 years old. The studied population respected the following criteria: no previous immune-related diseases, no HIV infection, no viral hepatitis and no history of immunosuppressive drug therapy, liver and kidney functioning normally, exclusion of other types of autoimmune diseases. Short-term administration of high-dose corticosteroids was reported to have a significant effect on AS patients, about improving their symptoms and influencing disease activity index. In order to exclude the effect of drugs on disease activity in AS patients, we selected newly diagnosed patients who were never medicated, did not receive a recent clinical drug treatment or short-term oral non-steroidal anti-inflammatory drugs (NSAIDs) such as cyclooxygenase (COX) inhibitors, glucocorticoids, anti-rheumatic drugs and was withoutanalgesics and opioids deliverywithin two weeks. All subjects had to fill in AS patient's questionnaire and Bath Ankylosing Spondylitis Functional Index (BASFI) under the guidance of laboratory personnel. According to Bath Ankylosing Spondylitis Disease Activity Index (BASDAI), the experimental population were divided into active groups (BASDI $\geq$ 4) and inactive group (BASDI < 4): 28 active patients counting for 21 males and 7 females; and 39 inactive patients divided in 32 males and 7 females. The healthy control group was composed of 30 individuals (24 males and 6 females, aged 19-52, average 27) whose blood samples were obtained from the Affiliated Hospital of Guiyang Medical College.

Blood specimen collection and samples preprocessing

Blood samples (2 ml) were collected from morning fasting subjects and put into blood collection tubes containing EDTA-2K. Samples were used forflow cytometry within six hours after acquisition.

For detecting the expression levels of TLR2 and TLR4 in peripheral blood of AS patients and healthy controls by flow cytometry, blood samples were preprocessed according to the procedure below. Antibodies were added in distinctively marked test tubes (TLR2-FITC in TLR 2/4 (+) and IgG1-FITC and IgG2a-PE in TLR 2/4 (-)). Following, 50 μL peripheral blood was introduced in the tubes and fully homogenized using a vortex, then incubated at room temperature away from light for 15 min. After that, 800 μL hemolysin was added and thoroughly mixed. Test tubes were incubated under light hemolysis avoidance for 3 min, and then transferred to 37°C water for 5 min. Thereafter, 1 mL PBS buffer was added to tubes, homogenized vigorously and centrifuged at 1000 rpm for 5 min. The supernatant was discarded and this step repeated once. After discarding the supernatant, suitable amount of PBS was added to samples and thoroughly mixed.

The same procedure was used for sample preparation of for the enumeration of lymphocytes subsets (CD19+CD5+, CD19+CD5-) except the used antibodies were dissimilar. Specifically, IgG1-APC and IgG2a-PE were added in isotype control tubes while CD19-APC and CD5-PE were added in CD19/CD5 (+) tubes.

For the detection of lymphocytes apoptosis, 50 μL blood specimens, 800 μL hemolysin and 1 mL PBS were mixed in two different tubes (one tube for control (apoptosis negative) and one testing tube (apoptosis+)) and homogenized vigorously. Following homogenization, tubes were centrifuged and washed once by discarding the supernatant. After centrifugation, 20 μL Annexin-V FITC, 20 μL PI and 50 μL Binding Buffer were added into the testing tubes (apoptosis +) and mixed vigorously while no antibody was added in the control tubes. Test tubes were incubated under light hemolysis avoidance for 3 min, and then transferred to 37°C water for 5 min. Thereafter, 1 mL PBS buffer was added to tubes, homogenized vigorously and centrifuged at 1000 rpm for 5 min. The supernatant was discarded and this step repeated once. After discarding the supernatant, suitable amount of PBS was added to samples and thoroughly mixed.

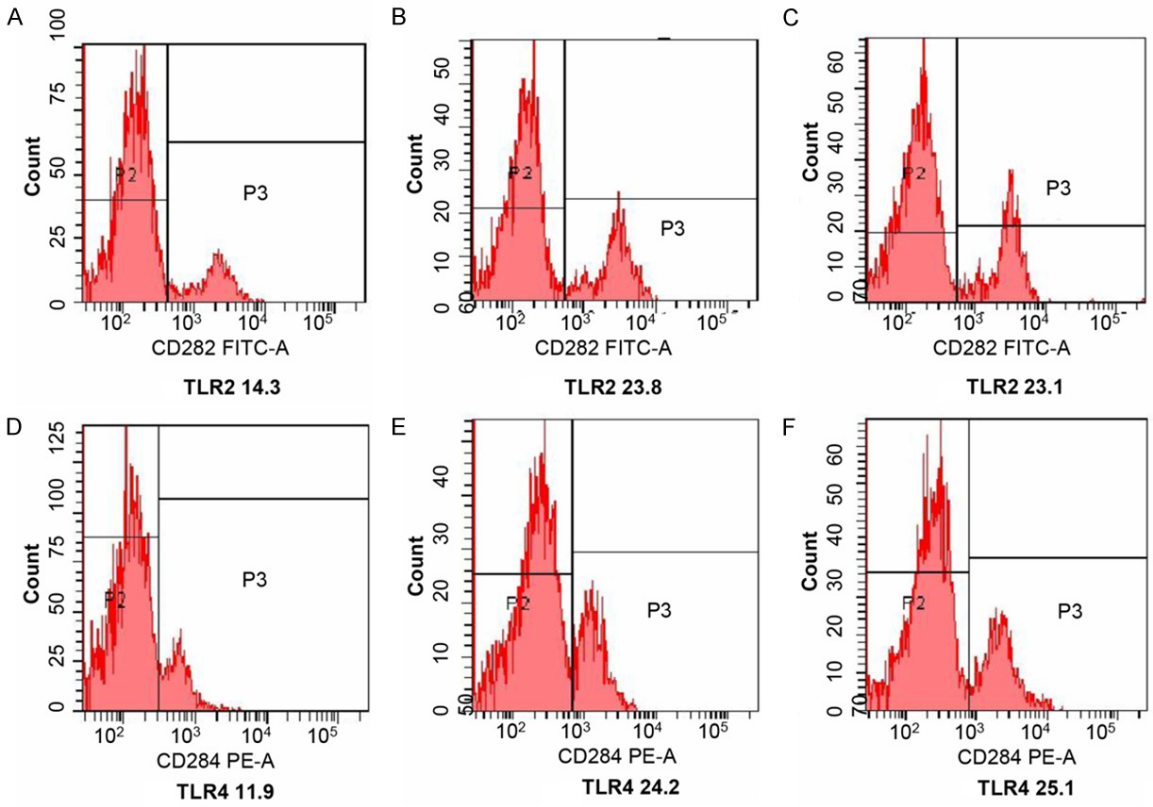

Figure 1. The flow cytometry chart obtained from the detection of the expression levels (%) of TLR2 and TLR4. A, D. Healthy control, B, E. Inactive AS patients, C, F. Active AS patients.

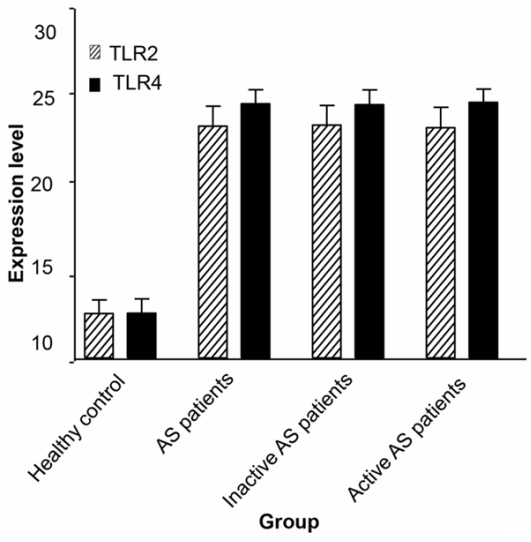

Figure 2. Expression levels (%) of TLR2 and TLR4 obtained from healthy control and different groups of AS patients.

Flow cytometry analysis

The suspensions were subjected to flow cytometry using FACS Canto II cytometer correctly adjusted to the best working state. The flow cytometer was run using the instrument operation boot program and preheated until warm-up. Standard calibration fluorescent microspheres (BD FACS 7-Color Setup Beads) were used for correcting the optical path and circuit, regulating the equipment voltage, predicting beads target value and testing the instrument sensitivity by means of FACS Canto software. Specimens (1×10^4 cells for each tube) were analyzed on the BD FACS Canto II cytometer using FACS Diva software.

Statistical analysis

Statistical analysis was performed using SPSS19.0 software. The experimental data were represented as mean ± standard deviation. Statistical analysis was carried out using variance analysis with $P < 0.05$ for detecting statistically significant differences between groups, and the least significant difference (LSD) method was adopted for multiple comparison analysis. In accordance with the normal distribution of the correlation analysis between two variables, we proceeded to statistical analysis by using Pearson correlation analysis method. If there was no conform normal

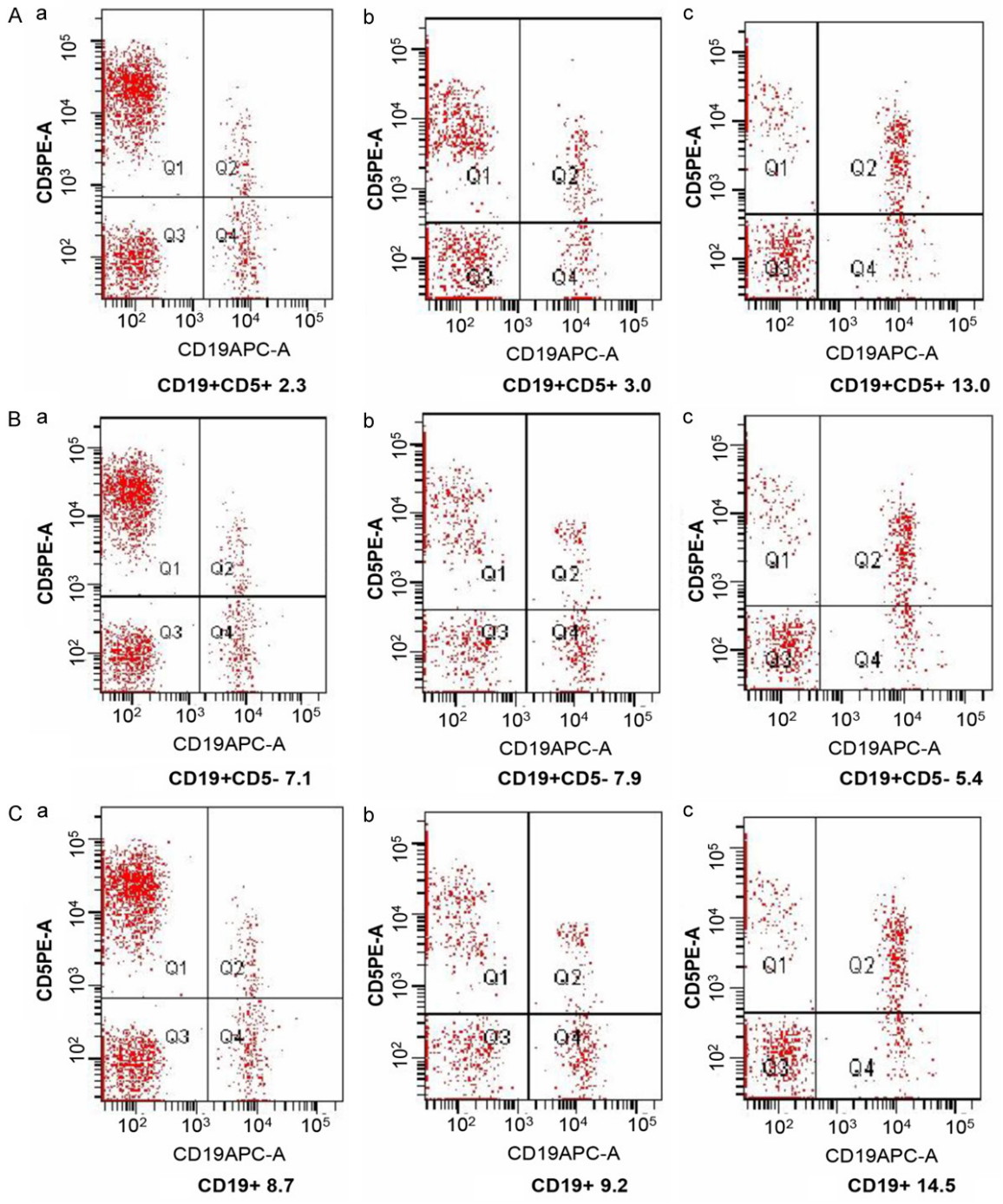

Figure 3. The flow cytometry chart obtained from the enumeration B lymphocytes subsets (A. CD19+CD5+, B. CD19+CD5-, C. CD19+). a. Healthy control, b. Inactive AS patients, c. Active AS patients.

distribution, we used Spearman correlation analysis method for studying the correlation between the two analyzed variables $P < 0.05$ as statistically different boundary value. $|r| > 0.7$ means high correlation between variables, $0.4 < |r| < 0.7$ means moderate correlation and $|r| < 0.4$ means low correlation.

Results

The expression levels of PBMC surface TLR2 and TLR4 in AS patients and healthy control

As shown in **Figures 1** and **2**, the expression levels of TLR2 and TLR4 in the PBMC of healthy

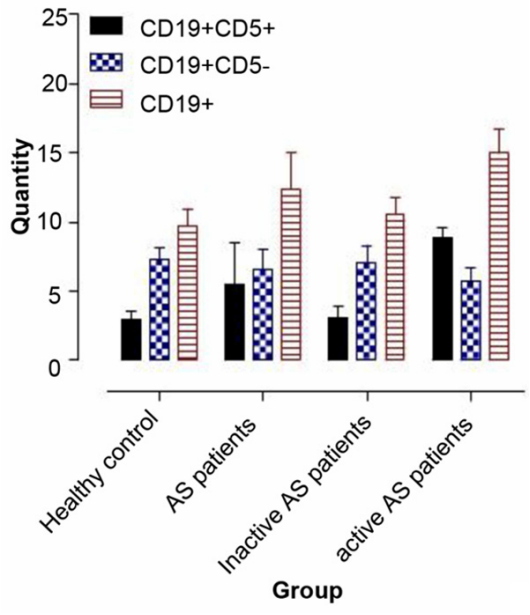

Figure 4. The flow cytometry chart obtained from the enumeration B lymphocytes subsets obtained from healthy controls and AS patients.

control group were 12.53% ± 0.77 and 2.55% ± 0.81, respectively. Compared to the control group, expression levels of TLR2 (23.16% ± 1.13) and TLR4 (24.43% ± 0.80) were higher in AS patient group, with a significant difference ($P < 0.01$). The increase of TLR2 and TLR4 expression levels suggested the involvement of the TLR signaling in the pathophysiology of ankylosing spondylitis. To investigate the prevalence of these molecules on the activity of the disease in the AS patients, we measured the expression levels in patients with active AS and those with inactive AS. The result demonstrated that the expression levels of TLR2 and TLR4 in inactive AS patients (n=39) were 23.22% ± 1.12 and 24.38% ± 0.84, respectively, and 23.07 ± 1.16 and 24.51 ± 0.75 in active AS patients (n=28), respectively, showing that there was no significant difference ($P > 0.05$) on the expression of these molecules between AS active and AS inactive patients. This suggested that, despite the impact of TLR2 and TLR4 signaling on the physiopathology of the AS disease, these molecules are not responsible for the disease activity in AS patients. In addition, we remarked similar expression level distribution of TLR2 and TLR4 within different studied groups suggesting that both molecules are identically expressed in the PBMC.

Enumeration of lymphocytes subsets CD19+CD5+ and CD19+CD5- in the PB of AS patients and healthy control

Different B lymphocytes subgroups CD19+ CD5+, CD19+CD5- and CD19+ B were enumerated using flow cytometry. **Figure 3** depicts the flow cytometry plots obtained from the enumeration of different subsets within the studied populations. The results of the quantification of B lymphocytes subsets were reported in **Figure 4**.

The result showed a significant ($P < 0.01$) increase in the number of CD19+CD5+ in AS disease group (5.50% ± 2.93) comparative to the healthy control group (2.97% ± 0.56). The counts of CD19+CD5+ in inactive AS group and active AS group were 3.11% ± 0.77 and 8.82% ± 0.75, respectively. No significant statistical difference ($P > 0.05$) was recorded between healthy control group and inactive AS group, whereas the difference among inactive and active AS groups was highly significant ($P < 0.01$). The above observations implied that AS is characterized by CD19+CD5+ lymphocytosis and CD19+CD5+ lymphocytosis seems to be in charge of the disease activity in AS patients.

The count of CD19+CD5- lymphocytes in AS patients (6.54% ± 1.44) was reduced in comparison with the healthy control group (7.28% ± 0.83). The difference was statistically significant ($P < 0.01$). The scrupulous analysis of CD19+CD5- lymphocytes within AS patients group revealed that the quantity of CD19+CD5- lymphocytes in inactive AS group (7.03% ± 1.28) was significantly ($P < 0.01$) higher than that in the active AS group (5.67% ± 0.99). Moreover, there was no statistically significant difference ($P > 0.05$) on the counts of CD19+ CD5- between patients with inactive AS and healthy controls. Characteristically, CD19+CD5- lymphocythemia was found to be a symptom associated with AS disease and its activity in AS patients.

We recorded a significantly high discrepancy ($P < 0.01$) on CD19+ B lymphocytes counts between AS disease group (12.40% ± 2.60) and the healthy control group (9.74% ± 1.20). The amounts of CD19+ B lymphocytes in inactive SA patients and active AS patients were 10.56 ± 1.15 and 14.96 ± 1.73, respectively. The difference among both groups was statisti-

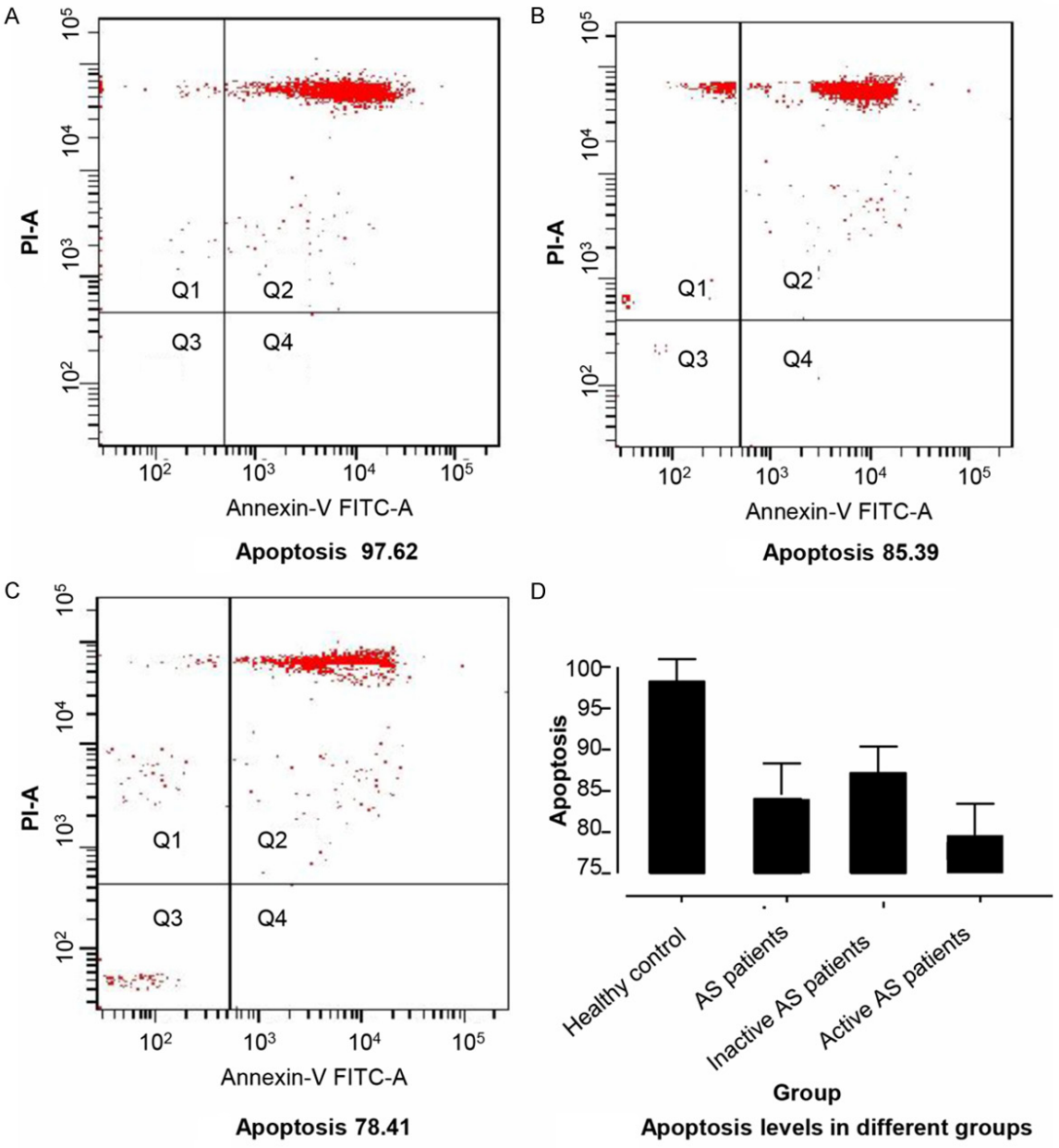

Figure 5. The flow cytometry chart and quantitative graph representation of lymphocytes apoptosis obtained from healthy controls and AS patients. A. Healthy control, B. Inactive AS patients, C. Active AS patients, D. Apoptosis levels in different groups.

cally significant, but the comparison of inactive AS group and healthy control group showed no statistically significant difference. Seemingly, CD19+ B lymphocytosis was found to be characteristic for AS disease and its activity.

Lymphocytes apoptosis in the PB of AS patients and healthy controls

The results of flow cytometry detection of lymphocyte apoptosis and its distribution within the studied groups is reported in **Figure 5**.

The results showed that lymphocyte apoptosis in the PB of AS disease group (84.00% ± 5.08) was reduced compared to the healthy control group (98.26% ± 0.70) with statistically significant difference ($P < 0.01$). Similarly, lymphocytes apoptosis in AS active group (79.64% ± 3.79) and AS inactive group (87.14% ± 3.23) was also reduced. The difference between AS active and inactive groups as well as that of the healthy control group with both groups were statistically significant ($P < 0.01$). This result demonstrated the dysregulation of apoptosis in

Table 1. Correlation between TLR2, TLR4, CD19+, CD19+CD5+ and CD19+CD5- with lymphocytes apoptosis in AS patients

		TLR2	TLR4	CD19+	CD19+CD5+	CD19+CD5-
Lymphocytes apoptosis	r-value	0.160	0.050	-0.736	-0.771	0.426
	P-value	0.099	0.345	0.000	0.000	0.000

Table 2. Correlation between TLR2, TLR4, CD19+, CD19+CD5+, CD19+CD5- and lymphocytes apoptosis with disease activity in AS patients

	TLR2	TLR4	CD19+	CD19+CD5+	CD19+CD5-	Lymphocytes apoptosis
r-value	0.043	0.112	0.709	0.757	-0.463	-0.568
P-value	0.730	0.367	0.000	0.000	0.000	0.000

AS patients. Specifically, the programmed cell death was decreased and probably led to an increase in lymphocytes number. In concordance with the lymphocytes enumeration results, cells likely to be touched by apoptosis dysregulation were CD19+CD5+ and CD19+ B lymphocytes.

Analysis of the correlation between TLR2, TLR4, CD19+, CD19+CD5+, and CD19+CD5- in the PB of AS patients and lymphocytes apoptosis

The result of the analysis of the correlation between TLR2, TLR4, CD19+, CD19+CD5+, CD19+CD5- in the PB with lymphocytes apoptosis is summarized in **Table 1**. The result showed no correlation between lymphocytes apoptosis and PBMC surface TLR2 (r=-0.160, $P > 0.05$) and TLR4 (r=0.050, $P > 0.05$). In AS patients, CD19+CD5+ (r=-0.771, $P < 0.01$) and CD19+ B lymphocytes (r=-0.736, $P < 0.01$) were negatively correlated with lymphocytes apoptosis. On the contrary, CD19+CD5- B lymphocytes and lymphocyte apoptosis were moderately and positively correlated (r=0.426, $P < 0.01$).

Analysis of the correlation between TLR2, TLR4, CD19+, CD19+CD5+, CD19+CD5- and lymphocytes apoptosis in the PB of AS patients and AS activity

The correlation analysis (**Table 2**) demonstrated that there is no correlation between the disease activity and toll-like receptors TLR2 (r=0.043, P > 0.05) and TLR4 (r=0.112, P > 0.05) while CD19+ B lymphocytes (r=0.709, P < 0.01) and CD19+CD5+ B lymphocytes (r=0.757, P < 0.01) in peripheral blood of patients were highly positively correlated with AS activity. AS patients PB lymphocytes apoptosis and CD19+CD5- B lymphocytes were moderately negatively correlated (r=-0.463, P < 0.01) with AS activity.

Discussion

Toll-like receptors (TLRs), belonging to the PRRs (pattern recognition receptors) family, have attracted major concerns in recent years as members of the innate immune molecules and become hot topics in up-to-date immunology research. A total of 10 kinds of TLR subtypes have been discovered so far and are likely to be involved in the pathogenesis of rheumatic diseases and are mainly expressed in immune cells such as dendritic cells, lymphocytes, monocytes and macrophages [20]. The autoimmunity is commonly connected with the pathogen-associated molecular patterns (PAMPs) and TLRs signaling, which, through the formation of PAMPs-TLRs complexes, causes the maturation of antigen presentation cells and the production of proinflammatory cytokines and chemokines, as well as plays an important role in body's natural immunity and acquired immunity [21-23]. Toll-like receptors (TLR) signaling is the body's first defense line against external infections. However, the excessive activation of signal cascade can break the body's immune tolerance to autoantigen and promote the development of autoimmune diseases. In the literature, it is reported that TLR is not only involved in antibacterial immunity but also in the occurrence of autoimmune diseases such as SLE and RA. TLRs identify specific patterns of microbial molecular components. Their discovery enabled the recognition of the innate and adaptive immunity and their mutual defense activity against invading patho-

gens through the effective connection of signal transduction. TLR subtypesare mainly involved in natural immunity. Among TLRs, TLR2 and TLR4 are the most widely expressed and are important molecules mediating innate and adaptive immune responses [24]. By stimulating T cell activation and proliferation, TLR2 and TLR4 play an important role in the pathogenesis of autoimmune diseases such as rheumatoid arthritis (RA) [25, 26]. The results of this study showed a significant increase of the expression of the PMBC surface TLR2 and TLR4 in AS patients compared with healthy individuals, which was corroborated with the findings of previous studies [27, 28]. Furthermore, the expression levels of TLR2 and TLR4 in active group and inactive AS patients groups were higher than those of the healthy control group , but the expression between the active and inactive groups was not statistically significant. The expression of TLR2 and TLR4 was not correlated with AS activity. The above results revealed abnormal changes of the expression profile of TLR2 and TLR4 in the pathogenesis of AS even at the inactivity stage, but this expression profile did not change with the emergence of disease abnormalities during the process of disease development. In the TLRs family, TLR2 and TLR4 express at cells surface, and are not only key molecules connected to both natural immunity and acquired immunity, but also major pathogen recognition receptors. TLR2 majorly recognizes lipoproteins and peptidoglycan of Gram-positive bacteria while TLR4 is mainly concerned in the identification of the lipopolysaccharide (LPS) of Gram-negative bacteria [29]. Infectious factors are suspected to play an important role in the etiology and pathogenesis of autoimmune diseases such as AS, and high expression of TLR2 and TLR4 might be related to the stimuli of infectious agents. Though there is no indication for the connection of TLR4 polymorphisms and mutations with the susceptibility to AS [30-33], studies showed the up-regulation of TLR4 in the PBMCs of AS patients [34] and TLR4 signaling was found to be strongly correlated with cytokines and disease activity parameters such as erythrocyte sedimentation rate (ESR) and plasma C-reactive protein (CRP). The fact that no association of TLR2 and TLR4 with AS activity was not observed in the present study suggesting that those molecules are not linked to AS activity or may indirectly intervene in the disease activity by activating a consortium of pro-inflammatory signaling pathways. The results of the present study revealed that, although there is no significant increase of the expression levels of TLR2 and TLR4 with the progression of the disease in AS patients, the up-regulation of these receptors in AS patients by comparison with healthy controls clearly displayed their involvement in AS pathogenesis.

B lymphocytes are key cells in humoral immunity. Wright, et al. [17] found that a variety of autoantibodies secreted by B lymphocytes could be detected in patients with AS, suggesting that B lymphocytes may be correlated with the pathogenesis of AS. In the pathogenesis of many autoimmune diseases, B lymphocytes play a key role in the abnormal proliferation or the production of autoantibodies. The results of this study showed significant increase of CD19+ B lymphocytes in the peripheral blood of AS patients compared to the healthy control group. CD19+ B lymphocytes were positively correlated with AS activity. This result suggested the presence of humoral immunity disorders in AS patients. In inactive AS patients CD19+ B lymphocytes did not show abnormalities, but the increase in disease activity was followed by the rise of abnormalities of CD19+ B lymphocytes which highly correlated with the disease activity, thus revealing the key role of humoral immunity in the process of development of AS disease. On the basis of the development sources, B cells can be divided into two classes B1 and B2. Besides, B1 cells can be divided into two subtypes B1a and B1b according to the ability or the inability to express CD5 molecules. CD19+CD5+ B cells as B1a cells expressing CD5 molecules are a class of B cells endowed of important functions in the maturation and self-stability of the immune system. The hyperproliferation of CD19+CD5+ B cells generally leads to a series of diseases [35]. Studies on CD19+CD5+ B lymphocytes in autoimmune diseases such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and several other studies found that CD19+CD5+ B lymphocytes in the peripheral blood of patients with these diseases were significantly increased. In this study, we similarly found that CD19+CD5+ B lymphocytes (B1a cells) in peripheral blood of AS patients were highly increased compared with the healthy control group, which was consistent with the increase

in CD19+ B lymphocytes. On the contrary, the amount of CD19+CD5- B lymphocytes (B1b cells, B2 cells) was lower than the healthy control group. This implies that abnormal changes in humoral immunity are directly related to the abnormal changes of B1a and B1b cells during the development of AS disease. In addition, there is no direct positive correlation between B2 cells and humoral immunity. The study also showed that CD19+CD5+ B lymphocytes were positively correlated with AS activity while a negative correlation was found for CD19+ CD5- B lymphocytes. The overexpression of CD19+CD5+ B lymphocytes and the down-regulation of CD19+CD5- B lymphocytes suggested that CD19+CD5+ B lymphocytes mediated immune responses during AS disease activity while CD19+CD5- B lymphocytes might play a role in the negative regulation of AS disease development. Constant levels CD19+ B lymphocytes and their subtypes were observed during the disease inactivity stage suggesting that the humoral immunity does not play a direct role during AS inactivity stage. The unusual large increase in Th1 in inactive AS group found in our preliminary study combined with the findings on TLR2 and TIR4 suggest that the occurrence of AS disease is intrinsically linked with cellular and innate immunity. The concomitant increase of CD19+ B lymphocytes and B1a cells and the reduction of the amount of B1b cells and B2 cells during the progression of the disease suggested that B1a cells play a dominant role in the development of humoral immunity whereas B1b cells and B2 cells might intervene in the negative regulation to probably maintain the body's immune homeostasis and immunity balance. During disease activity stage, the significant reduction in B1b cells and B2 cells might be associated with the increase in B1a cells and there may exist a cross- regulation between B1a cells and B1b and B2 cells. The hyperexpression of B1a cells resulted in attenuated expression of B1b cells and B2 cells. This caused the enhancement of the function of regulation of CD19+ B lymphocytes which secreted large amounts of autoantibodies leading to autoimmune diseases.

In recent years, a large number of studies have shown that the lack of proliferation and apoptosis about T cells are related to increased occurrence of certain autoimmune diseases [36]. Apoptosis, also known as programmed, is the physiological state of the cell death by the initiative of genes. Apoptosis and the immune system is particularly close since apoptosis is not only involved in the growth and development of immune cells, immune regulation, immune effector and multiple physiological functions, but also plays an important role in the maintenance of the stability of immunological functions [37]. The results of the study showed low quantity of apoptotic lymphocytes in the peripheral blood of AS patients compared with healthy control group, reflecting the slowdown of lymphocytes apoptosis in AS. This result suggested the existence of an intrinsic relationship between AS and lymphocytes apoptosis. The results of the study also showed that lymphocyte apoptosis and AS disease activity was negatively correlated and the number of apoptotic lymphocytes in the active group and inactive group were both lower than the healthy control group, suggesting that lymphocyte apoptosis is less involved in AS autoimmune disease. Lymphocytes apoptosis in active and inactive group of AS patients was also statistically significantly different, suggesting that lymphocytes apoptosis was maintained abnormal over the disease development and severely affected tissue metabolism. The number of apoptotic lymphocytes decreased with the progression of disease activity. This down-regulation of lymphocytes apoptosis led to the relative increase of corresponding lymphocytes. These dysregulations concordantly resulted in autoimmune disorders and abnormal expression of the immune response in AS patients. Numerous studies suggested that TLR2 signaling pathway is mainly involved in immune function and inflammation reactions, including inflammatory cell activation and apoptosis [38, 39]. The implication of TLR2 in the mediation of apoptosis abnormal changes has not been reported in AS disease development. In this study, we found that lymphocytes apoptosis in AS patients is not associated with the expression levels of TLR2 and TLR4 but further study is needed to dissect the specific reasons of this statement. However, we also found that CD19+ B lymphocytes, CD19+CD5+ B lymphocytes and lymphocyte apoptosis changes were negatively correlated while CD19+CD5- B lymphocytes and apoptosis were positively correlated, suggesting that during the development of AS, CD19+ B lymphocytes and CD19+CD5+ B lymphocytes may be major factors causing

abnormal reduction of lymphocytes apoptosis. During AS disease activity, CD19+ B lymphocytes and CD19+CD5+ B lymphocytes were significantly increased in comparison with the healthy control group, while the number of apoptotic lymphocytes during this period was significantly reduced. The rate of apoptosis had the tendency to slow down with the progression of the disease. Apoptosis is an important form of the maintenance of immune tolerance and immune balance which are generally the targets of autoimmune diseases.

The results of the study showed that the dysregulation of lymphocytes apoptosis has a certain influence on the immune function in patients with AS. The normal physiological lymphocytes death is suppressed in patients with AS, leading to the perpetual survival of lymphocytes that should be normally cleared and this may be correlated with the longevity of CD19+ CD5+ B lymphocytes. Aberrant mechanisms of the clearance of apoptotic immune cells in AS patients conducted to the destruction of immunity equilibrium, the dysfunction of immune system homeostasis, the provision of conditions favorable for the occurrence of hypersensitivity and abnormal relative increase of immune cells resulting into the production of a large number of autoantibodies and the induction of the occurrence and development of autoimmune diseases. The occurrence of the development of AS is not closely related to the apoptosis of lymphocytes which may be caused by abnormal expression of B lymphocytes during the disease.

Conclusively, AS patients showed some abnormal changes concerning the expression levels of mononuclear cell surface TLR2 and TLR4, the count of B lymphocyte subsets and the number of apoptotic lymphocytes during the occurrence and development of AS disease. During AS inactivity period, the expression levels of mononuclear cells surface TLR2 and TLR4 can be used to detect the differential diagnosis of AS and could be of fundamental significance for the discovery and treatment of the disease. CD19+ B lymphocytes and each subset can be used to evaluate the extent of disease progression in patients; it can also be used for detecting the development of disease and timely adjustment of medication in order to reduce the suffering and morbidity of patients. We also found a close relationship between lymphocytes apoptosis reduction and abnormal increase of various subsets of B lymphocytes.

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Disclosure of conflict of interest

None.

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