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Dynamic Changes of CD16+ Monocytes in Ankylosing Spondylitis and Its Significance

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Abstract: Background: The pathogenesis of ankylosing spondylitis (AS) is unclear and the incidence has been increasing in recent years. Objective: To explore the trend and clinical significance of peripheral blood mononuclear cell subtypes in patients with AS. Method: Detect plasma levels of seven cytokines (TNF-α, IL-2, IL-4, IL-6, IL-10, IL-17A, and IFN-γ), analyze the correlation between the percentage of CD16+monocyte subtypes and cytokine expression at the same level. Result: Compared with the control group, the plasma levels of five cytokines in the AS group were significantly higher than those in the control group ($P<0.05$), and were positively correlated with the content of CD16+monocytes. Conclusion: CD16+monocytes are closely related to the occurrence of AS; Detecting CD16+monocyte subtypes in patients' peripheral blood can provide new experimental indicators for the diagnosis of AS. Taking AS as a starting point, it provides a new research direction for the treatment and prognosis detection of other autoimmune diseases in the future.

Keywords: Ankylosing Spondylitis, Monocyte Subtype, Cytokines, Flow Cytometry

1. Introduction

Ankylosing Spondylitis (AS) is a chronic disease with the main pathological changes of the spine. It is caused by genetic and environmental factors. It mainly involves the spine and sacroiliac joints, causing spinal ankylosis and difficulty in movement [1]. It can also cause eye, lung, cardiovascular, kidney and other organ damage in different degrees. Ankylosing Spondylitis can be treated by drugs, surgery and physical methods [2] At present, it can not be cured and needs lifelong intermittent treatment. The disease is a polygenic genetic disease. The main susceptible gene of AS is HLA-B27 [3-5]. The pathogenesis of AS is complex. Many mechanisms indicate that stimulate inflammation and immune response, cause tissue damage and participate in the occurrence and development of the disease [6, 7]. recent studies have shown that monocyte subtypes play a key role in autoimmune diseases and inflammation. In this study, the percentage of peripheral blood mononuclear cell subtypes and the content of cytokines TNF-α, IL-2, IL-4, IL6, IL-10, IL-17a, and IFN-γ were detected in patients with AS, and the correlation between the percentage of CD16+ monocyte subtypes and cytokine expression level was analyzed, and the clinical significance of peripheral blood monocyte subtype detection in AS was explored.

2. Materials and Methods

2.1. Materials

2.1.1. Research Object

Methods: from October 2018 to October 2020, 50 patients with ankylosing spondylitis in the Affiliated Hospital of Beihua University were selected, including 35 male and 15 female cases, and the ratio of male to female was 7:3; The age ranged from 21 to 74 years, with an average of (43.24±15.01) years. 40 patients with chronic inflammatory arthritis were recruited into the study; Diagnosis of AS was based on the modified New York criteria [8], All the patients were positive for HLA-B27 [9] by flow cytometer (FCM) There were 40 cases of HLA-B27 negative samples provided by the health examination center of the hospital and tested by FCM, including 26 males and 14 females, with a male to female ratio of 13:7; The mean age was (45.15±18.17) years (range, 21-79 years). The patients and the control groups were comparable

in age, gender and other natural conditions. Clinical characteristics of recruiting patients did not show any baseline differences between groups. This study was approved by the medical ethics committee of the Affiliated Hospital of Beihua University, and the participants were informed and agreed to provide peripheral blood for the experimental study.

2.1.2. Main Instruments and Reagents

Flow cytometry (Beckman Coulter company, USA); enzyme labeling instrument (Beijing Pulang Technology Co., Ltd.); -80°C ultra-low temperature refrigerator (Haier company); vertical centrifuge (tg16m, Changsha Yida Instrument Co., Ltd.); two color fluorescence labeled monoclonal antibodies HLA-B27-FITC / HLA-B7-PE; monochromatic fluorescent labeled monoclonal antibodies CD14-PE and CD16-FITC (Beckman Coulter, USA); Human plasma cytokines TNF-α, IL-2, IL-4, IL-6, IL-10, IL-17A and IFN-γ detection kits (Jiangxi Saiji Biotechnology Co., Ltd.).

2.2. Methods

2.2.1. Detection of HLA-B27 in Peripheral Blood

Add 5 μL HLA-B27-FITC / HLA-B7-PE double color fluorescent labeled monoclonal antibody into the special test tube of FCM, then add 30 μl EDTA-k2 anticoagulant whole blood, gently mix and mark for 15min at room temperature. Add 600 μl hemolytic agent A, shake for 8 s, immediately add 260 μl hemolytic agent B, and shake well. Add 7 ml PBS, 1100 r / min centrifugation for 5 min, discard the supernatant, leave the cells precipitated at the bottom, and shake with 2 ml PBS buffer. After mixing, HLA-B27 was detected by FCM.

2.2.2. Detection of the Percentage of Monocyte Subtypes in Peripheral Blood

The monoclonal antibodies labeled with 5 μl CD14-PE and 5 μl CD16-FITC were added into the special test tube of FCM, and then the whole blood was labeled with 30 μl EDTA-k2. After dissolving the red blood cells (the method is the same as above), FCM was used for detection. The monocytes were set up with CD14-PE as the abscissa and CD16-FITC as the ordinate. The percentages of classical monocytes (CD14 + + CD16 -), intermediate monocytes (CD14 + CD16 +) and non-classical monocytes (CD14-CD16 + +) were observed.

2.2.3. Detect the CD85K Percentage of CD16+ Monocytes

5μl CD85K-PE, CD3-PC5 and CD14-CD16-FITC fluorescent labeled monoclonal antibody were added into the special test tube of FCM, and then 30 μl EDTA-K2 was added to anticoagulant whole blood for labeling. After dissolving red blood cells (the method is the same as above), FCM was used to detect monocytes. The expression level of CD85k in CD16+ monocytes was determined by setting up a coordinate system with CD14-PE as the horizontal coordinate and CD85K-APC as the vertical coordinate.

2.2.4. Detect the Plasma Cytokines

Venous blood samples were collected with EDTA anticoagulant tube and centrifuged at 3000 rpm for 10 minutes. About 0.5ml plasma was collected. Calculate the required number of people n; open the quantitative standard (b), Transfer the standard substance into a centrifuge tube and mark the tube as the maximum concentration; suspend the standard sample with 2ml sample diluent (g) and place it at room temperature for 15min; mix the standard sample with a suction head gently to avoid violent vibration; take 10 experimental sample tubes and mark them as 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, and add 300ul of sample diluent (g) into each tube Extract 300ul liquid into 1:2 tube, blow and mix, take 300ul liquid from 1:2 tube into 1:4 tube, blow and mix, and so on until 1:512 tube; centrifuge the capture microsphere mixture (a) with low-speed centrifuge 200g for 5min, carefully suck out the supernatant, add the same volume of microsphere buffer solution (H) as the aspirated supernatant, and incubate for 30min away from light after the vortex is fully mixed In each tube, 25ul of standard substance with gradient dilution was added into the standard tube; 25ul of sample to be tested was added to each tube of sample tube; 25ul of fluorescent detection reagent C was added to all test tubes; All test tubes were fully mixed and incubated in dark at room temperature for 2.5 hours; 1ml PBS buffer was added into each tube of the test tube, and 210g centrifugation was conducted for 5 minutes, and the supernatant was discarded; each tube was added with 100ul PBS buffer solution and stood for detection. The plasma levels of TNF-α, IL-2, IL-4, IL-6, IL-10, IL-17A and IFN-γ were measured by FCM.

2.2.5. Statistical Analysis

SPSS17.0 was used for statistical analysis. The measurement data were expressed as mean ± standard deviation ($\bar{X} \pm SD$) and t-test was used; Pearson method was used to analyze the correlation between the two variables, and the difference was statistically significant with $P < 0.05$.

3. Results

3.1. Flow Cytometry Was Used to Detect HLA-B27

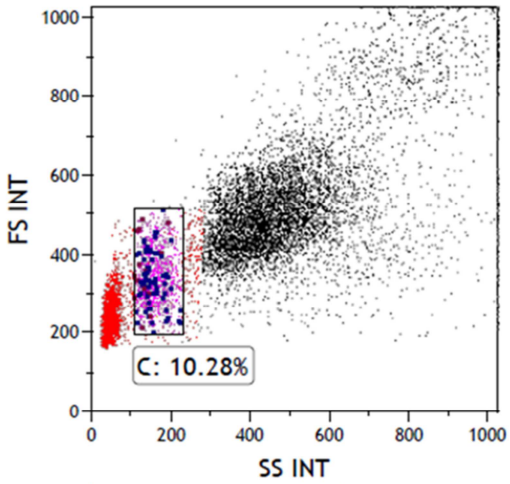
HLA-B27-FITC-HLA-B7-PE fluorescent labeled monoclonal antibody was used to detect the positive rate and the average fluorescence intensity of HLA-B27. The positive criteria of HLA-B27 were as follows: the positive rate was more than 80%, and the average fluorescence intensity was more than 8. The detection results of as samples met the above criteria. All 50 patients in the AS group had positive HLA-B27, while all 40 patients in the control group had negative HLA-B27. The natural information is shown in Table 1.

Table 1. *Natural Information Sheet.*

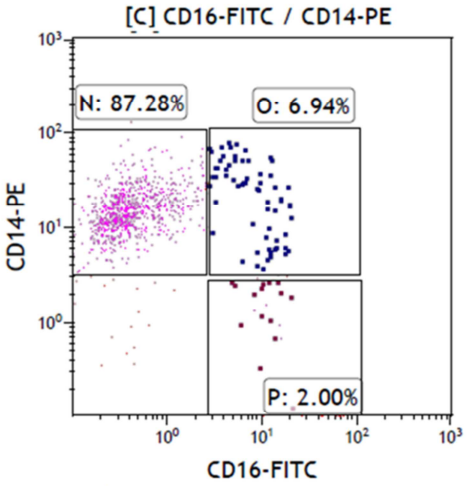
	AS	CTrOL	P Value
Demogragpic Factors			
Age (X ± SD)	43.24±15.01	45.15±18.17	>0.05
Gender (%)			
Male (n)	70% (35)	65% (26)	>0.05
Femal (n)	30% (15)	35% (14)	>0.05
HLA-B27+(n)	50	0	

3.2. Detect the Percentage of Peripheral Blood Mononuclear Cell Subtypes in AS Group and Control Group

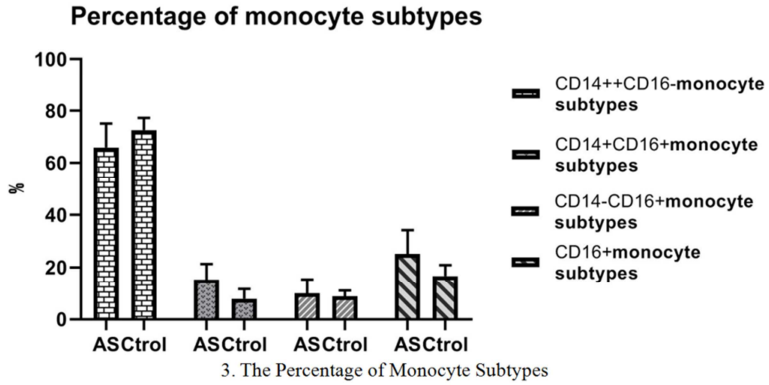
Flow cytometry was used to determine the percentages of three subtypes of monocytes in peripheral blood of 50 patients with AS, 40 normal controls. The results were shown in Graph 1. The percentage of CD16+ monocytes (CD14- CD16+ and CD14+ CD16+) in AS group was significantly higher than that in the control group, and the difference was statistically significant ($P < 0.05$). The percentage of typical mononuclear cells (CD14++ CD16-) was lower than that of the control group, showing no statistical significance ($P > 0.05$).



1. The Peripheral Blood Mononuclear Cell Area Was Mapped with Portal Scatter Plots



2. Detection of CD14 and CD16 Subtypes by Flow Cytometry (CD16+ Monocytes: P+O).



3. The Percentage of Monocyte Subtypes

Figure 1. *Flow Cytometry Detection Diagram.*

3.3. Detect the Percentage of CD85K on the Surface of CD16+ Monocytes (CD14+CD16+ and CD14-CD16+) in AS Group and Normal Control Group

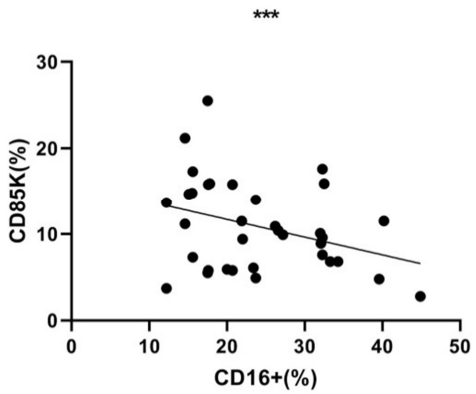


Figure 2. *Linear relationship Between CD85K Percentage and CD16+ Monocytes (***: p<0.015).*

Flow cytometry was used to determine the percentage of CD85K on the surface of CD16+ monocytes (CD14+CD16+ and CD14-CD16+) in the peripheral blood of 50 AS patients and 40 normal controls. The results were shown in Graph 2. The percentage of CD85K in CD16+ monocytes (CD14+CD16+ and CD14-CD16+) in AS group was significantly higher than that in the control group, and the difference was statistically significant ($P < 0.01$).

3.4. Detect Plasma Cytokine Levels in AS Group and Normal Control Group

Plasma levels of five cytokines (TNF-α, IL-6, IL-10, IL-17A and IFN-γ) in the AS group were higher than those in control group ($P < 0.05$). The levels of TNF-α, IL-6, and IFN – γ in AS group were significantly higher than those in control group ($P < 0.01$). The results were shown in Graph 3. (There was no statistically significant difference in IL-2 and IL-4 between the two groups, and the ancient results were not shown.)

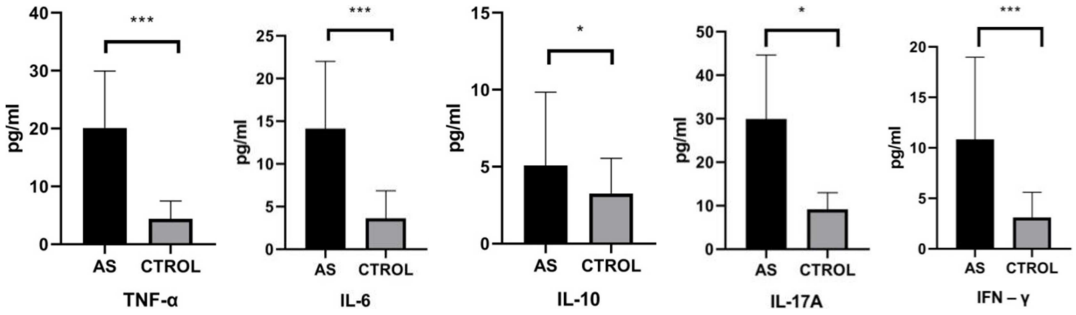


Figure 3. *SPSS Software T-test Diagram of Cytokines in AS Group and Control Group (***: p<0.01;*: p<0.05).*

3.5. Correlations Between the Percentage of CD16+ Monocyte Subtypes and the Levels of Three Cytokines in AS Group

Pearson correlation test showed that the percentage of peripheral blood CD16+ monocytes (CD14 + CD16 +and CD14-CD16+) was positively correlated with the expression levels of plasma cytokines TNF-α, IL6 and IL-17A 50 AS patients, and the correlation coefficients (r) were 0.4151, 0.6011 and 0.1631 (all P < 0.01). The results were shown in Graph 4.

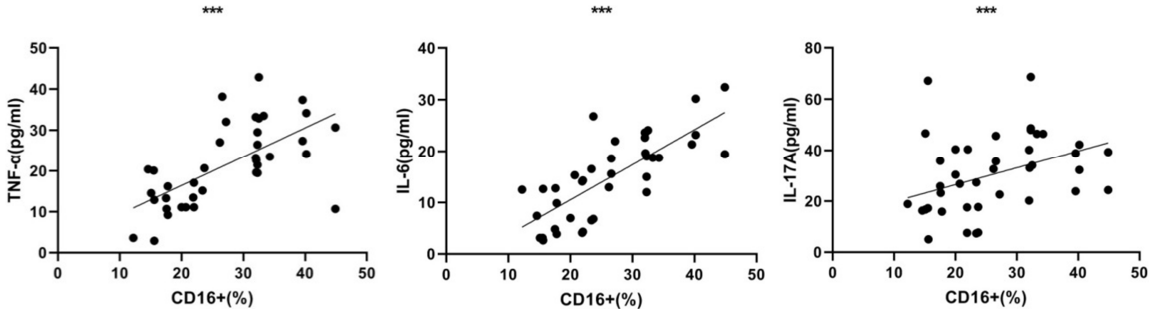


Figure 4. *Linear relationship Between Cytokine Content and Percentage of CD16+ Monocytes (***: p<0.01).*

4. Discussion

The pathogenesis of ankylosing spondylitis (AS) is closely related to the function of immune cells and the expression of proinflammatory cytokines. As an important immune cell, monocytes play an important role in the process of AS. Monocytes are mononuclear cells developed from bone marrow stem cells. They can stay in the blood circulation system, or enter the tissues and organs to further develop into macrophages. A large number of studies have shown that monocytes have obvious heterogeneity, with the most significant differences in phenotype, function and inflammatory activation protein. Activated monocytes on their surface exhibit upregulated antigenic expressions such as CD14, CD16, HLA-Dr, toll-like receptors (TLrs), and adhesion molecules B1 and B2 integrins [10]. The difference of expression levels of CD14 and CD16 can be divided into different subtypes. According to the Nomenclature Committee of the international society of immunology, Mononuclear cells were divided into three subgroups according to their surface markers CD14 and CD16: classical type (CD14+ +CD16 -), middle type (CD14+ CD16 +) and atypical type (CD14-CD16 ++), which accounted for about 85%, 5% and 10% of peripheral blood mononuclear cells, respectively. Different subgroups showed significant differences in phenotype, function and inflammatory activation potential [11]. Some experiments have shown that mature macrophages express CD16, along with low levels of CD14, which can be distinguished from CD14++ monocytes. These indicate that CD16+ cells are more mature cells than CD14++ monocytes.

The percentage of monocyte subtypes in peripheral blood of patients with AS was detected by flow cytometry, the proportion of CD16+ monocytes (CD14 + CD16 + and CD14-CD16+) increased in AS. Classical monocyte pool is primarily skewed towards CD16+ monocytes and the stimulating factor for the differentiation is M-CSF [12, 13]. Moreover, the predominant intermediate monocytes in AS differentiate into M1-macrophages which play a major role in synovial inflammation. It plays an important role in autoimmune diseases.

Foreign studies have shown that CD16 + monocytes play an important role in the progression of a variety of immune and infectious diseases, such as rheumatoid arthritis, diabetes, tuberculosis, etc [13]. Kawanaka et al. [14] previously reported that elevated CD16 expressing monocyte subsets are characteristic for autoimmune diseases and the active disease state. Melgert et al. [15] showed that the proportion of typical monocytes in peripheral blood of patients with preeclampsia decreased, while the proportion ofCD16+ monocytes

increased. Studies on some other inflammatory diseases have also confirmed that CD16+ monocytes play an important role in the process of various autoimmune diseases, including rheumatoid arthritis, atherosclerosis, Kawasaki disease, septic shock, multiple sclerosis, type 1 diabetes, etc [16, 17]. Therefore, an increase in CD16+ monocytes may be a common phenomenon in autoimmune and infectious diseases.

The phagocytic ability of classical monocytes (CD14 + + CD16 -) was the strongest; non-classical monocytes (CD14-CD16 + +) highly express cytoskeleton movement related genes, which show that they can crawl along the inner wall of blood vessels and exhibit unique "patrol" function, which is reflected in their ability to recognize inflammatory signals, migrate rapidly to inflammatory sites, and rapidly differentiate into macrophages after infiltrating tissues [18, 19]; CD16+ monocytes (CD14 + CD16 + and CD14-CD16++) can quickly recruit to the inflammatory site, and generate a large number of pro-inflammatory factors, which has a stronger potential to activate the inflammatory response and participate in the inflammatory pathological process. CD16+ cells are increasingly present in both blood and synovium of autoimmune diseases patients, where they may express autoantigens correlating with the severity of joint damage [20].

5. Conclusion

Monocytes can migrate to different tissues and differentiate into tissue-specific macrophages or dendritic cells. Dendritic cells are the APC with the strongest antigen-presenting function, which can stimulate the activation and proliferation of initial T cells. It has been shown that when CD16+ monocytes were cultured for 2 days in the presence of TLr2 ligands, they would preferentially develop into CD16+ dendritic cells with higher antigen presentation capacity [21].

CD85K antigen is a member of immunoglobular like transcription (ILT), is a transmembrane protein of 60kDa, with 2 extracellular lg-SF domains, and 3 cytoplasmic ITIMs which is selectively expressed on myeloid antigenpresenting cells and up-regulated on tolerogenic DCs [22-24], expressed in monocytes, macrophages, dendritic cells and granulocytes. Studies have shown that CD85k antigen participates in the uptake and presentation of antigen, and negatively regulates the functional response of antigen presenting cells (APC) by stimulating receptors such as CD16 and CD11b [25-27]. Importantly, CD85k also marked a Treg subset with superior suppressive capacity toward Th1 but not Th17 effector cells. while CD85k might play a more important role in the resolution of the effector response and possibly also in promoting memory formation [26]. CD16+ monocytes may fail to cross endothelial barriers at the onset of inflammation, while differentiating in peripheral blood and tissues due to perturbed cytokine production from classical CD14+CD16− subsets [28]. Monocyte adhesion and activation markers are other emerging fields of interest. CD11b is involved in migration and adhesion. Polymorphisms of CD11b are associated with systemic lupus erythematosus and other immune complex mediated diseases [29].

HLA-Dr is MHC-II type of molecule, contains two relative molecular mass of 36 respectively of 36,000 and 27, 000 subunits (alpha and beta subunits). HLA-Dr is expressed on the surface of antigen-presenting cells (such as monocytes, macrophages, B lymphocytes, etc.) and activated T lymphocytes. Antigen-presenting cell (APC) HLA-Dr plays an important role in the process of antigen presentation to CD4 + T cells and activates the adaptive immune response. Monocytes have a long half-life and their HLA-Dr expression is relatively stable [30, 31].

In this study, it was found that there was no significant difference in HLA-Dr between typical and non-typical monocytes of the control group. Because the proportion of CD16+ monocytes in peripheral blood monocytes is low, even if the expression of HLA-Dr in CD16+ monocytes is increased, it is difficult to have an obvious influence on the mean value of HLA-Dr in overall monocytes. CD16+ monocytes showed higher HLA-Dr levels than CD14++CD16- monocytes, indicating higher antigen presentation ability of this group of cells.

CD16+monocyte subsets (CD14+ CD16+ and CD14-CD16+) which produce proinflammatory cytokines such as TNF-α, IL-6, and IL-17a. Some scholars [32] have used inflammatory antigen lipopolysaccharide (LPS), staphylococcus enterotoxin (SEB) and peptide polysaccharide (PGN) to stimulate the subjects and then take whole blood for culture. The changes of TLr2 and TLr4 were then detected by flow cytometry. The results showed that tumor necrosis factor –α (TNF-α) and interleukin-6 (IL-6) were increased 2 h after stimulation. CD16+ mononuclear cell ratio increased at 20 h, and the ratio was positively correlated with TNF-. When intracellular cytokine staining was performed on monocytes, it was found that the stimulation of TLr-4 ligand LPS and TLr-2 ligand Pam3Cys in whole blood showed a higher level of TNF expression in CD16+ monocytes. CD16+ monocytes showed higher levels of TNF and IL-6 production compared with CD14++CD16- monocytes [33]. TNF-α is a typical pro-inflammatory cytokine that plays an important biological role in promoting apoptosis [34]. IL-10 is a typical anti-inflammatory factor, playing a role of down-regulating inflammatory response and antagonizing inflammatory mediators. Studies have found that IL-10 can inhibit the synthesis of TNF-α at the transcriptional level [35]. Normal levels of IL-10 is extremely low, will increase after the stimulus, by some factors in mononuclear cells in patients with cirrhosis after the stimulation of endotoxin, inflammatory factor will be increased, including proinflammatory factor increased more obviously, and IL-10 anti-inflammatory factor is increasing little or not, which can lead to disorder of pro-inflammatory/anti-inflammatory balance, this performance is more outstanding in the CD16 + mononuclear cells. The high expression of these pro-inflammatory cytokines TNF-α leads us to believe that CD16+ monocytes are a group of pro-inflammatory monocytes.

Regulatory T cells (Treg) and helper T cells 17 (Th17) are CD4 + T cell subsets that have been extensively studied in

recent years. The proportion or functional imbalance of Treg /Th17 cell subsets plays an important role in the occurrence and development of AS, Th17 is a novel cell subgroup of CD4+T cells, which is characterized by the secretion of IL-17a and IL-22 and plays an immunosuppressive role [36]. As a pre-inflammatory cytokine, IL-17a can activate the biological activities of nK-KB and MAPKs kinases in cells by binding to IL-17 receptors on the cell surface of the body, mediating the accumulation of neutrophils and macrophages at the inflammatory sites, causing the infiltration and tissue destruction of inflammatory cells, and participating in the maturation, proliferation and chemoattractant of neutrophils [37]. IL-17A induces autoimmune diseases [38, 39]. IL17A can recruit and activate neutrophils, induce T cells to activate and stimulate epithelial cells, macrophages, and fibroblasts to produce inflammatory factors, such as TNF-, IL-6, IL-8, IL-1, etc., thereby inducing inflammation [40]. Studies [41] have shown that increased IL-17A is associated with a variety of chronic inflammatory diseases.

The over-activation of Th17 cells releases a large amount of inflammatory mediators such as IL-17A, which may increase the progression of disease by enhancing the inflammatory response [41], thereby mediating the occurrence and development of AS. IL-17 can promote the activation of T cells and stimulate the production of various cytokines by epithelial cells, endothelial cells and fibroblasts, such as IL-6, IL-8, granulocyte-macrophage stimulating factor (GM-CSF), chemical activators and cell adhesion molecule-1. These cytokines can act alone or in collaboration, leading to the production of inflammation [42]. The results of this study showed that serum IL-17a level in the AS group was significantly higher than that in the normal control group, and the increased level was positively correlated with the upregulation of the percentage of CD16+ monocytes. It was further confirmed that CD16+ monocytes play an important pro-inflammatory role in the course of AS disease. The results of this experiment showed that IL-2 and IL-4 showed no statistically significant difference between the two groups, so relevant discussions were not conducted in this paper.

This study showed that the proportion of classic monocytes in peripheral blood of AS decreased, while the proportion of CD16+ monocytes increased, and related inflammatory cytokines were released, resulting in the high expression of TNF-α, IL-6, IL-17A and IFN – γ in peripheral blood. It is suggested that in AS, the proportion of monocytes subtypes has changed, that is, the proportion of monocytes is polarized to CD16+ monocytes, which can be inferred as CD16+ monocytes participate in the course of AS through immune and inflammatory response related pathways, and are more likely to infiltrate into the inflammatory tissue, making it in a sustained inflammatory state, aggravating the disease. It has clinical reference value for the diagnosis of AS, and also provides new ideas for the study of the pathogenesis of AS, inhibition of intermediate polarization of monocytes is expected to be a new target for the drug therapy. In conclusion, this group of experiments showed that the number of CD16 + monocytes increased significantly in patients with AS and was related to abnormal expression of cytokines, suggesting that CD16 + monocytes may participate in the occurrence and development of diseases by regulating the expression of cytokines, which provided a new idea for understanding the immunological pathogenesis of AS. However, this study has not yet clarified the specific mechanism by which CD16 + monocytes regulate the expression of related cytokines. In the future, animal models and in vitro experiments should be used to further clarify its specific mechanism, so as to lay a foundation for its application in clinical disease monitoring in the future.

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