Original Article

The immune dysfunction in ankylosing spondylitis patients

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Summary   Ankylosing spondylitis (AS) is a spinal arthritic disease that is often associated with human leukocyte antigen (HLA)-B27, while only part of HLA-B27 carriers become AS patients. T cells have been reported to play an important role in the pathology of AS. T-cell immunoglobulin and mucin-domain-containing molecule 3 (Tim-3) and programmed death-1 (PD-1) have been known to negatively regulate the immune response. In this study, we used flow cytometry to analyze the immunological differences of peripheral blood from 21 patients with AS, 22 cases who didn't have AS but were found to be HLA-B27 positive (HLA-B27+ group), and 16 normal healthy individuals (Healthy group). The level of $CD4^+$, $CD8^+$ T cells, and Treg of each group was observed. The expression of Tim-3 and PD-1 and the production of IFN-γ, IL-6, TNF-α, IL-4, and IL-10 were examined as well. We found that the percentage of Treg in AS group was lower than that of healthy group. The expression of PD-1 on $CD8^+$ T cells and Tim-3 on $CD4^+$ T cells was lower in the AS group. AS group had lower IL-10 production by $CD4^+$ T cells and higher IL-6 production by $CD8^+$ T cells. The results of HLA-B27+ group were similar to that of the healthy group. These data suggested that patients with AS had an impairment in the ability to negatively regulate the immune response, which might be related to the etiology of AS. To further investigate the roles of Tim-3 and PD-1 on $CD4^+$ T cells and $CD8^+$ T cells, we tested IFN-γ and IL-10 production by $Tim-3^+$, $Tim-3^-$, $PD-1^+$, and $PD-1^-$ $CD4^+$ and $CD8^+$ T cells from AS patients. We found that $Tim-3^+CD4^+$, $Tim-3^+CD8^+$ and $PD-1^+CD8^+$ T cells produced more IL-10 than other subsets. In conclusion, there is a dysfunction of T cells in AS that is associated with PD-1 and Tim-3.

Keywords: PD-1, Tim-3, IL-6, IL-10, $CD4^+$ T cell, $CD8^+$ T cell, Ankylosing spondylitis

1. Introduction

Ankylosing spondylitis (AS) is a chronic inflammatory disease whose etiology remains unclear (*1*). The treatment takes a long time and requires control of the associated inflammation. It often affects sacroiliac joints, the spine and peripheral joints (*2*).

Recent studies perceive AS as an autoimmune disorder that is associated with human leukocyte antigen (HLA)-B27 (*3*) and T cells (*4*). T cells appear to take part in maintaining immune tolerance in humans, whose dysfunction leads to diseases like systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), recurrence spontaneous abortion (RSA) and some inflammatory bowel diseases (*5-8*). The alternations of T cells found in AS patients include increased frequency of peripheral Th2 lymphocytes (*9*) and Th17 lymphocytes (*10-11*). $CD4^+$ T cells may play an important role in the pathology of AS, so as $CD8^+$ T cells (*12*). Moreover, it is noteworthy

that spondyloarthropathies can be recognized by the presence of CD4^{+} T cells and HLA-B27 reactive CD4^{+} T cells may be related to these disorders (*13*). Of interest, only part of the HLA-B27 positive carriers become AS patients.

In addition to T cells, the inhibitory co-stimulatory molecules PD-1 and Tim-3 have also been a hot topic in AS research. Programmed death-1 (PD-1), first reported in 1992, belongs to immunoglobulin (Ig) superfamily (*14*) and can be found on the surface of peripheral T cells, B cells, NK cells, dendritic cells, and monocytes (*15*). PD-1 maintains immune tolerance and protects tissues from autoimmune attack by acting with its ligand (PD-L) (*16*). T-cell immunoglobulin and mucin-domain-containing molecule 3 (Tim-3), often expressed in Th1, Th17, and CD8^{+} T cells (*17*), has been known to downregulate immune responses and control inflammation (*18*). PD-1 and Tim-3 are often found to have unusual expression in chronic infections and autoimmune diseases, including HIV infection (*19*), tuberculosis (*19*), sepsis (*20*), and feto-maternal immune regulation (*21*).

In the course of immunity and defense, the delicate balance between IFN-γ and IL-10 is crucial. IFN-γ is an activator of T cells, monocytes, and neutrophils, which drives the clearance of pathogens, while IL-10 acts to hinder this process (*22-24*).

To explore the etiology and pathology of ankylosing spondylitis, we designed this experiment to make a further step in understanding the influence of immune tolerance in AS. AS patients, HLA-B27 positive and HLA-B27 negative normal healthy subjects were recruited. The level of CD4^{+}, CD8^{+} T cells, and Treg of each group was observed by flow cytometry. The expression of Tim-3 and PD-1 was examined and the production of IFN-γ, IL-6, TNF-α, IL-4, and IL-10 was tested as well. To further investigate the roles of Tim-3 and PD-1 on CD4^{+} T cells and CD8^{+} T cells, we tested IFN-γ and IL-10 production by Tim-3^{+}, Tim-3^{-}, PD-1^{+}, and PD-1^{-} CD4^{+} and CD8^{+} T cells from AS patients.

Our study aimed at investigating the frequency change of T cells in AS patients and determining the expression and production of these inhibitory co-stimulatory molecules and regulatory molecules in the development of AS. Most importantly, it was of clinical significance to make a comparison of AS patients, HLA-B27 positive, and HLA-B27 negative normal healthy subjects, which offered a new perspective to have an overall examination of the screening index HLA-B27.

2. Materials and Methods

2.1. *Reagents*

Flow cytometry antibodies CD4-FITC, IL-6-PE, CD8-FITC, TNF-α-PE/cy7, IL-10-BV421, Tim-3-PE, IL-4-APC, PD-1-APC, CD25-PE, CD127-APC, IFN-γ-PE/cy7, as well as Fix/Perm Buffer, were purchased from Biolegend (San Diego, CA, USA).

2.2. *Patients and controls*

Table 1 showed the information of all the studied subjects. A total of 21 AS cases (14 men and 7 women) were included in this study, along with 22 cases (14 men and 8 women) who didn't have AS but were found to be HLA-B27 positive (HLA-B27+) in a conventional medical examination, and 16 healthy controls (11 men and 5 women) who were selected from Ren Ji Hospital of Shanghai Jiao Tong University. The AS patients, aged 16-63 years, were diagnosed by the modified New York Criteria (*25*). Disease activity was evaluated using the Bath AS Disease Activity Index (BASDAI) (*26*), with the 21 patients' BASDAI Score > 4. There was no significant difference in percentage of gender and age among three groups.

2.3. *Flow cytometry (FCM)*

Peripheral blood (PB) was harvested in the presence of EDTA-K$_2$. One aliquot of PB was incubated with the monoclonal antibody CD4-FITC for 30 minutes, after which red blood cell lysis buffer was added. The sample was washed with PBS, then placed in Fix/Perm Buffer for 40 minutes to lyse the white blood cells, and then washed again with PBS. The antibodies IL-4-APC, IL-6-PE, and TNF-α-PE/cy7 were added and incubated for 30 minutes, and the sample was then washed with PBS.

Table 1. Clinical characteristics of AS, HLA-B27+, and Healthy groups

Items	AS (*n* = 21)	HLA-B27+ (*n* = 22)	Healthy (*n* = 16)
Age			
Range (years)	16-63	17-64	20-62
Mean ± SD	37.0 ± 9.8	38.3 ± 14.0	34.6 ± 10.1
Gender, n (%) male	14 (66.67)	14 (63.64)	11 (68.75)
Clinical features			
HLA-B27 positive	all	all	none
BASDAI	> 4	NA	NA

NA: not available.

A second aliquot of PB was incubated with monoclonal antibodies against CD4, PD-1, and Tim-3 for 30 minutes, after which red blood cell lysis buffer was added. The sample was washed with PBS, then placed in Fix/Perm Buffer for 40 minutes to lyse the white blood cells and washed again with PBS. Antibodies against IFN-γ and IL-10 were added and incubated for 30 minutes, and the sample was then washed with PBS.

Another aliquot of PB was treated similarly as above, except CD8 antibody used instead of CD4 antibody, to test IL-4, IL-6, TNF-α, PD-1, and Tim-3 on it.

Last aliquot of PB was stained by CD4-FITC, CD25-PE, and CD127-APC to test Treg.

Approximately 100,000 stained cells in each sample were analyzed by a CyAN ADP flow cytometer (Beckman Coulter, Brea, CA, USA).

2.4. *Statistical analysis*

All values were expressed as the mean ± SD. Data was analyzed with SPSS ver.19 and Prism 5. Demographic data among the groups was compared by the Chi-square test. Differences were considered significant at $p < 0.05$.

3. Results

3.1. *AS patients showed lower Tim-3 on CD4$^+$ T cells*

The percentage of CD4$^+$ T cells was of no significant difference among three groups (Figure 1B). Compared with the healthy group (1.39 ± 0.69%), the AS group (0.94 ± 0.47%) showed notably lower ($p < 0.05$) Tim-3$^+$/CD4$^+$ (Figure 2A). However, the percentage of PD-1$^+$/CD4$^+$ was not significantly different among three groups (Healthy, 32.67 ± 6.27%; HLA-B27+, 37.75 ± 19.25% *vs.* AS, 32.78 ± 12.18%) (Figure 2B). These data suggested the decline of Tim-3 expression on CD4$^+$ T cells in AS patients while CD4$^+$ T cells and PD-1 remained unchanged.

3.2. *AS patients showed lower PD-1 on CD8$^+$ T cells, while Tim-3 is unchanged*

The percentage of CD8$^+$ T cells in the three groups was also analyzed by flow cytometry, and no significant difference was found among these groups (Figure 1D). We found obviously lower expression of PD-1$^+$/CD8$^+$ in the AS group (22.04 ± 8.25%) than the healthy group (35.92 ± 6.21%) and the HLA-B27+ group (31.93 ± 13.16%) (Figure 2D). However, the percentage of Tim-3$^+$/CD8$^+$ was not significantly different (10.91 ± 3.74, 10.27 ± 4.85 *vs.* 11.48 ± 5.01) (Figure 2C). The results suggested the downregulation of PD-1 expression on CD8$^+$ T cells in AS patients while CD8$^+$ T cells and Tim-3 remained unchanged.

3.3. *Expression of IL-10 in CD4$^+$ T cells was lower in AS patients*

To further explore the downstream changes in AS develoment, we examined IFN-γ, IL-6, TNF-α, IL-4, and IL-10 expression to analyze the intracellular events. We found that there was no significant
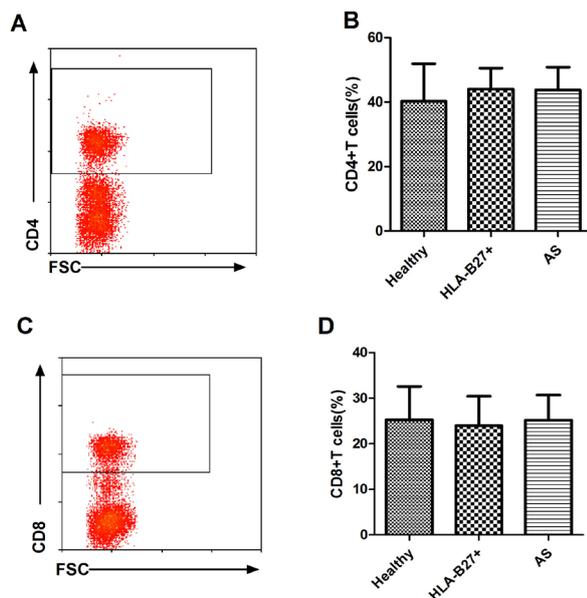
Figure 1. The percentage of CD4$^+$ and CD8$^+$ T cells in different groups. Samples were analyzed by flow cytometry with antibodies against CD4 and CD8 marking the T cell surface and with gating for CD4- and CD8-positive lymphocytes. Data was presented as the mean ± SD. The analysis compared the AS group ($n = 21$) with the healthy group ($n = 16$) and the HLA-B27+ group ($n = 22$).
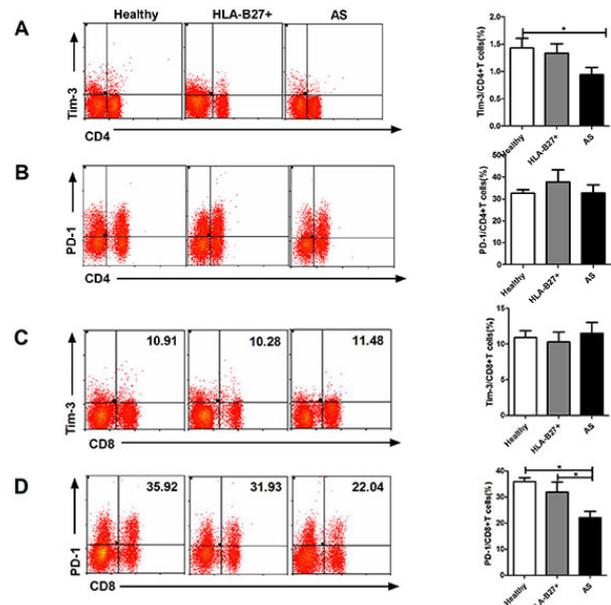
Figure 2. The expression of Tim-3 on CD4$^+$ and PD-1 on CD8$^+$ T cells in AS patients. Blood samples from the AS group ($n = 21$), the healthy group ($n = 16$) and the HLA-B27+ group ($n = 22$) were analyzed by flow cytometry. **(A, B)** CD4$^+$ T cells positive for Tim-3 and PD-1, analyzed by flow cytometry. **(C, D)** CD8$^+$ T cells positive for Tim-3 and PD-1, analyzed by flow cytometry. Data was presented as the mean ± SD. *$p < 0.05$.

difference in the expression of IFN-γ/CD4+ among three groups (healthy, $86.56 \pm 13.12\%$; HLA-B27+, $91.53 \pm 6.50\%$; and AS, $85.55 \pm 8.69\%$). Similar results were obtained for IL-6, TNF-α, and IL-4 in CD4+ T cells. However, IL-10/CD4+ showed a decrease in the AS group. The results in Healthy, HLA-B27+, and AS group were $1.92 \pm 0.94\%$, $1.79 \pm 0.87\%$, and $0.91 \pm 0.51\%$, respectively (Figure 3).
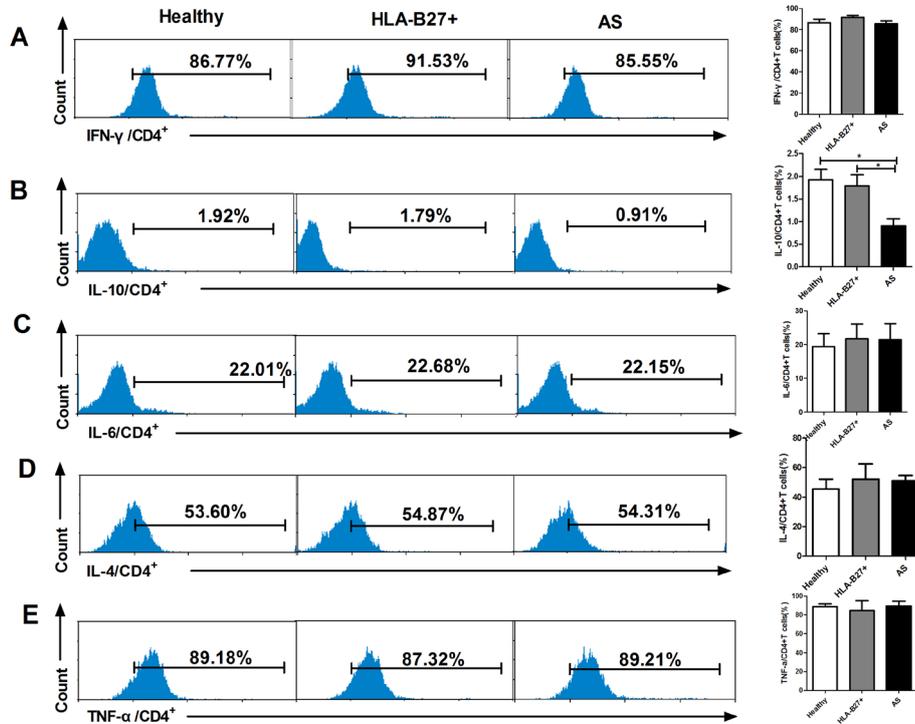
Figure 3. The production of cytokines in CD4+ T cells in different groups. CD4+ T cells were analyzed by flow cytometry for the production of IFN-γ, IL-6, TNF-α, IL-4, and IL-10. Data was analyzed using Prism 5 **(B)**.*$p < 0.05$, AS ($n = 21$), Healthy ($n = 16$) and HLA-B27+ ($n = 22$).
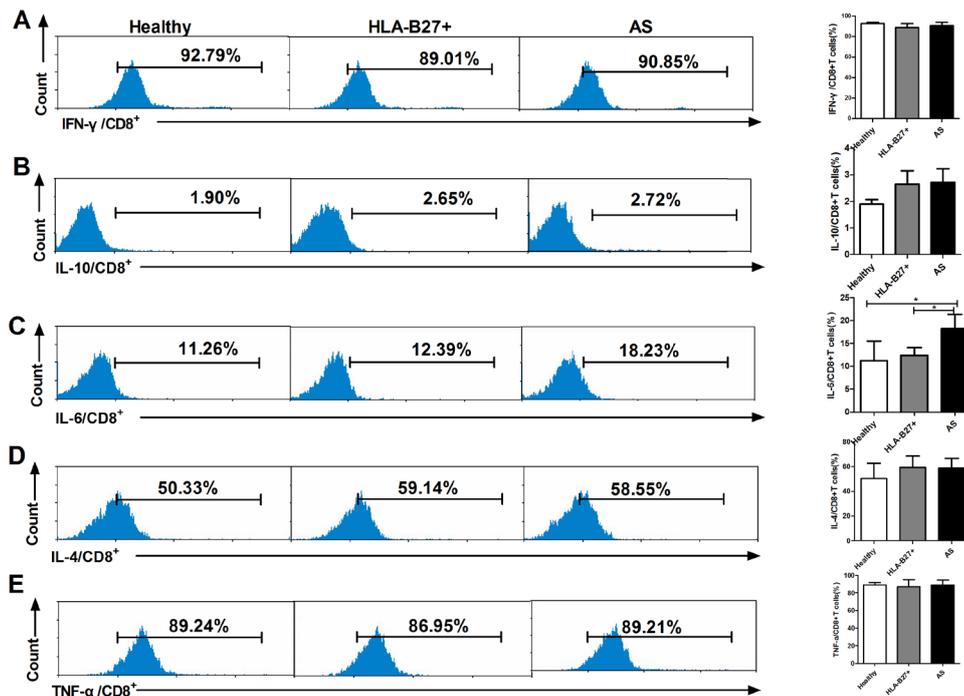
Figure 4. The production of cytokines in CD8+ T cells in different groups. The production of IFN-γ, IL-10, TNF-α, and IL-4 in CD8+ T cells did not differ among three groups, while IL-6 was higher in AS patients. Data was analyzed using Prism 5. *$p < 0.05$, AS ($n = 21$), Healthy ($n = 16$) and HLA-B27+ ($n = 22$)

3.4. Expression of IL-6 in CD8⁺ T cells was higher in AS patients

We also tested the expression of IFN-γ, IL-6, TNF-α, IL-4, and IL-10 in CD8⁺ T cells. The frequency of IL-6/CD8⁺ cells in the AS group ($18.23 \pm 0.94\%$) was higher than the healthy group ($11.26 \pm 1.28\%$) and HLA-B27+ group ($12.39 \pm 0.63\%$). However, other cytokines showed no significant differences among three groups (Figure 4). These results suggested higher expression of IL-6/CD8⁺ in AS patients.

3.5. Tim-3⁺ T cells from AS patients produced more IL-10

To further investigate the roles of Tim-3 and PD-1 on CD4⁺ T cells and CD8⁺ T cells, we tested IFN-γ and IL-10 production by Tim-3⁺CD4⁺, Tim-3⁻CD4⁺, Tim-3⁺CD8⁺, Tim-3⁻CD8⁺, PD-1⁺CD4⁺, PD-1⁻CD4⁺, PD-1⁺CD8⁺ and PD-1⁻CD8⁺ T cells from AS patients. We found that Tim-3⁺CD4⁺, Tim-3⁺CD8⁺ and PD-1⁺CD8⁺ T cells produced more IL-10 than other subsets of T cells (Figure 5). There was no obvious difference in IFN-γ production. These data suggested that PD-1 and Tim-3 might influence the production of IL-10.

3.6. AS patients showed lower percentage of Treg

Table 2 showed the percentage of PBMC in AS,

HLA-B27+, and Healthy groups. The percentage of CD4⁺CD25⁺CD127^low T cells in these three groups was also analyzed (Figure 6). We found that the percent of CD4⁺CD25⁺CD127^low/CD4⁺ in AS patient ($2.70 \pm 0.80\%$) was significantly lower than the healthy group ($3.47 \pm 0.83\%$) ($p < 0.05$), but there was no significant difference compared to HLA-B27+ group ($2.96 \pm 0.35\%$) ($p > 0.05$).

4. Discussion

Ankylosing spondylitis is a spinal arthritic disease that is often associated with HLA-B27 (27-29); however, fewer than 5% of HLA-B27 carriers become AS patients (30-31), which leaves over a lot of questions. We started from the immunological differences among AS patients and both HLA-B27 positive and negative healthy controls to further explore the immune regulation in the development of AS, with focus on Tim-3 and PD-1.

We first determined the total levels of CD4, CD8 and Treg lymphocytes among three groups. The results turned out that there was no significant difference in the expression of either CD4⁺ T cells or CD8⁺ T cells, while AS patients showed lower percentage of Treg. Our finding of CD4⁺ T cells is similar to the results of Aktas *et al.* (*32*) but in contrast to the research of Chen *et al.*

Figure 5. Tim-3⁺ and PD-1⁺ T cells produced more IL-10 than Tim-3⁻ and PD-1⁻ T cells. We examined IFN-γ and IL-10 production by Tim-3⁺CD4⁺, Tim-3⁻CD4⁺, Tim-3⁺CD8⁺, Tim-3⁻CD8⁺, PD-1⁺CD4⁺, PD-1⁻CD4⁺, PD-1⁺CD8⁺, and PD-1⁻CD8⁺T cells from AS patients ($n = 21$). Tim-3⁺CD4⁺, Tim-3⁺CD8⁺ and PD-1⁺CD8⁺ T cells produced more IL-10 than other subsets of T cells. *$p < 0.05$.
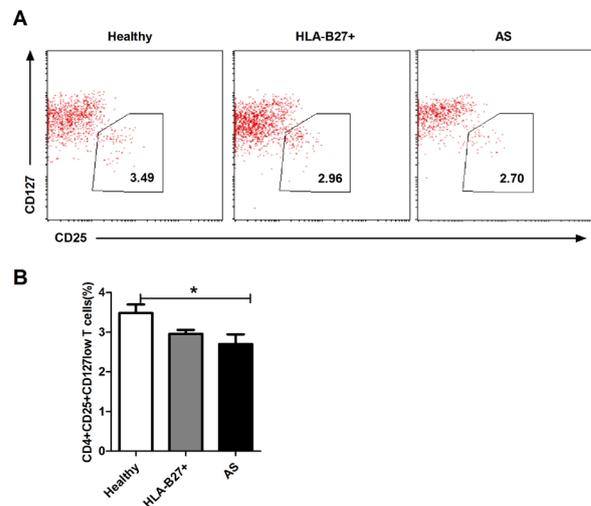
Figure 6. The expression of CD4⁺CD25⁺CD127^low T cells in AS patients. The percentage of CD4⁺CD25⁺CD127^low T cells in three groups was analyzed by flow cytometry. We found that it was significantly lower than healthy group ($p < 0.05$), but of no significant difference than HLA-B27+ group. Data was analyzed using Prism 5. *$p < 0.05$, AS ($n = 21$), Healthy ($n = 16$) and HLA-B27+ ($n = 22$).

Table 2. Percentage of PBMC in AS, HLA-B27+, and Healthy groups

Items	AS ($n = 21$)	p value	HLA-B27+ ($n = 22$)	p value	Healthy ($n = 16$)
CD3⁺ T cells	63.21 ± 7.46	0.42	57.85 ± 9.16	0.38	60.42 ± 10.52
CD4⁺ T cells	40.33 ± 2.53	0.29	44.04 ± 1.40	0.20	43.76 ± 1.72
CD8⁺ T cells	25.18 ± 1.37	0.96	24.00 ± 1.37	0.54	25.29 ± 1.59
CD4⁺25⁺127^low T cells	2.70 ± 0.80	0.03	2.96 ± 0.35	0.33	3.47 ± 0.83

p value: *vs.* Healthy group.
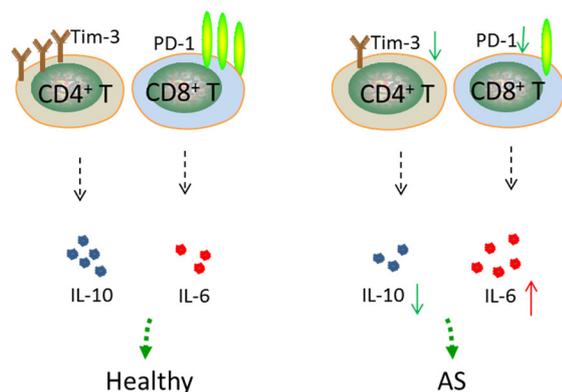
Figure 7. Schematic diagram of PD-1 and Tim-3 signals in AS patients. The expression of Tim-3 on CD4$^+$ T cells and PD-1 on CD8$^+$ T cells in AS was lower, accompanied by inadequate production of cytokines IL-10, which suggested poor inflammation inhibitory. The number of PD-1 on CD8$^+$ T cells in AS was decreased while their production of cytokines IL-6 was higher, which possibly contributed to the occurrence of AS.

(*33*). We speculated that it was due to the small sample and different severity of disease. The C-reactive protein of AS patients in Chen's report was 1.84 ± 0.41mg/dL, while we didn't detect it. The level of CD8$^+$ T cells, which recognize MHC 1 molecules directly to defend against infections, was also not higher in the AS patients. This results indicated that changes of number in CD4 and CD8 lymphocytes are not associated with the occurrence of AS.

Tim-3 and PD-1 take part in control negative regulation of immune responses in many diseases (*34*), establishing the balance between the activation and inhibition of T cells, with a tendency towards the latter. We found that the expression of Tim-3 on CD4$^+$ T cells and PD-1 on CD8$^+$ T cells was notably lower in AS, which implied that Tim-3 and PD-1 on T cells could affect the disease.

Cytokine analysis showed that IFN-γ, IL-6, TNF-α, and IL-4 levels in CD4$^+$ T cells were not altered, but IL-10 level in CD4$^+$ T cells was lower in the AS group. As a classic immuno-regulatory and anti-inflammatory cytokine, IL-10 had decreased expression in CD4$^+$ T cells which might correlate with AS. Meanwhile, IL-6 level in CD8$^+$ T cells was obviously higher in the AS group than the other two control groups. Proinflammatory cytokine IL-6 is known to induce the activation of osteoclasts (*35*), which might closely relate to the bone metabolism associated with AS.

Taken together, our study showed that the dysfunction of immune suppression played an important role in AS. A paradigm that illustrated the possible mechanism of Tim-3 and PD-1 signals in regulation of the immune response in AS was proposed (Figure 7).

Our study did not succeed in finding the immunological differences between the HLA-B27 positive and negative healthy groups, which almost shared the same expression levels of the tested markers. This might explain the reason why less than 5% of HLA-B27 positive individuals develop AS (i.e., most HLA-B27 positive individuals do not have downregulated Tim-3 and PD-1 expression). Tim-3$^+$CD4$^+$, Tim-3$^+$CD8$^+$, and PD-1$^+$CD8$^+$ T cells were found to produce more IL-10 than other subsets of cells (Tim-3$^-$CD4$^+$, Tim-3$^-$CD8$^+$, and PD-1$^-$CD8$^+$ T cells). Therefore, we hypothesized that Tim-3 and PD-1 were involved in the development of AS through the regulation of IL-10 production. The role of Tim-3 and PD-1 on CD4$^+$ and CD8$^+$ T cells should be investigated further to examine the pathogenesis of AS.

Our findings provided a new way to think about the diagnosis and treatment of AS. Current pharmacological therapies and physiotherapies, such as regular exercise or spa therapy (*36*), mainly relieve the symptoms of AS (*36*). Dorothea *et al.* reported that body hyperthermia treatment could increase IL-10 and toll-like receptor 4 expression which were believed to play an active role in the curative effect (*37*). In recent studies, blockade of PD-1 or Tim-3 molecules to restore the balance of immune regulation has been a hot topic (*23,38*). The development of this blockade as a way to ameliorate AS is the basis of our ongoing work.

Acknowledgements

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