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Assay of Peripheral Regulatory Vδ1 T Cells in Ankylosing Spondylitis and its Significance

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Background: Ankylosing spondylitis (AS) involves inflammation at the sacroiliac joint and spine attachment site. This study aimed to observe the ratio and function of peripheral regulatory Vδ1 T cells in AS patients to investigate their roles in AS pathogenesis.

Material/Methods: Peripheral blood mononuclear cells (PBMC) were separated by density-gradient centrifugation from AS patients and healthy controls. Flow cytometry was used to determine the ratio between Vδ1 and CD4 T cells of PBMC in AS patients and controls. Flow cytometry sorting (FCS) was used to obtain Vδ1 and naïve CD4 T cells with purity higher than 90%. CFSE staining method was used to detect the effect of Vδ1 T cells on proliferation of naïve CD4 T cells. The effect of Vδ1 T cells on secretion of IFN-γ from naïve CD4 T cells and the ability to secrete IL-10 from Vδ1 T cells were determined by flow cytometry.

Results: AS patients had significantly lower Vδ1 T cell ratio in PBMC compared to controls ($p<0.05$), but their CD4 T cell ratio was significantly elevated ($p<0.05$). Functional assay showed suppression of naïve CD4 T cell proliferation and IFN-γ secretion by peripheral Vδ1 T cells in AS patients ($p<0.01$). AS patients also had lower IL-10 secreting level from peripheral derived Vδ1 T cells ($p<0.01$).

Conclusions: The immune suppression of peripheral Vδ1 T cell in AS patient increases the ratio of peripheral CD4 T cells and IFN-γ level, leading to AS pathogenesis. This immune suppression is mainly due to suppressed IL-10 secretion.

MeSH Keywords: Alcoholic Neuropathy • BALB 3T3 Cells • Hyperostosis, Diffuse Idiopathic Skeletal • Interferon-alpha

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Background

Ankylosing spondylitis (AS) is a bone disease involving inflammation at the sacroiliac joint and spine attachment site; it is also a type of rheumatic disease and is strongly correlated with HLA-B27 [1,2]. AS is also a chronic inflammatory disease involving tissue fibrosis and bone transformation in the fibrous ring of intervertebral discs and nearby tissues, and is manifested as joint stiffness. Its clinical features mainly consist of inflammation at the sacroiliac joint and spine attachment site, thus causing bone formation at the lesion site [3–6]. Little is known regarding the exact cause of AS pathogenesis. It has been shown that certain microbes (e.g., *Klebsiella bacillus*) share similar antigens with susceptible individuals, thus causing abnormal immune response. Previous studies showed the close correlation between imbalance of body immune regulation and the occurrence/progression of AS [7,8]. Treatment of AS using anti-TNF-α works by counter-acting over-secreted TNF-α, in addition to down-regulating peripheral Th17 ratio and related cytokines, as well as up-regulating CD4 Treg cell ratio [8]. However, other immune-suppressing cells, such as regulatory B cells, regulatory DC cells, and Vδ1 T cells, also exist in peripheral blood along with CD4 Treg [9–11]. Vδ1 T cells have been recently demonstrated to have immune-suppressing functions [11], mainly by secreting IL-10. However, the ratio and function of Vδ1 T cells in peripheral blood of AS patients have not been studied. We thus observed the changed cell ratio of Vδ1 T cells in AS patients, and also investigated their functional changes, in an attempt to illustrate the correlation between Vδ1 T cells and progression of AS.

Material and Methods

Clinical information

A total of 50 AS patients (42 males and 8 females, age 28.53±8.15 years) in Qilu Hospital, Shandong University from 30 Oct 2013 to 30 Dec 2014 were recruited in this study, along with a control group of 50 healthy individuals (42 males and 8 females, age 27.93±8.52 years) who came to the hospital for general physical examinations. All AS patients were HLA-B27-positive, and fit the AS diagnosis guideline (New York, 1984). No treatment had been received by any AS patients before the study.

The experimental protocol was pre-approved by the Ethics Committee of Qilu Hospital, Shandong University and written consent was obtained from all patients and healthy volunteers.

Peripheral blood mononuclear cell (PBMC) collection

Fasting blood samples were collected from all patients on the next morning after being admitted, and from controls on the morning of the day they received their physical examination. Density-gradient centrifugation was used to separate PBMCs, as previously described [12]. In brief, fasting blood samples were collected in sterile anti-coagulant tubes with an equal volume of PBS. The mixture was then added into a centrifuge tube containing 15 mL of lymphocyte separation buffer (2:1 ratio) for 800 g centrifugation for 18 min. The white layer on top was saved and mixed with 10 mL of sterile PBS, which was then centrifuged at 600 g for 15 min. Cell precipitation was re-suspended in 10 mL of sterile PBS, and was centrifuged at 400 g for 8 min. One mL of sterile PBS was then used to re-suspend cell precipitation. After Trypan blue quantification, cells were prepared for suspensions at 2×10^6 per mL.

Vδ1 T cells/CD4 T cell ratio assay

PBMCs were rinsed twice in PBS containing 5% BSA. After removing supernatants, cells were incubated with antibody (FITC-Vδ1, APC-CD3, PE-CD4) at 4°C for 30 min. Cells were then rinsed twice in PBS containing 5% BSA. After removing supernatants, cells were re-suspended in 0.1 mL PBS for assay.

Vδ1 T cell immune-suppressing function assay

Flow cytometry sorting (FCS) method was used to obtain Vδ1 T cells and naïve CD4 T cells with purity higher than 90%. In the assay of naïve CD4 T cell proliferation by Vδ1 T cell inhibition, a 96-well plate was pre-coated with 1 μg/mL CD3 antibody and 2 μg/mL D28 antibody, followed by 37°C incubation for 2 h. Naïve CD4 T cells were stained by CFSE method following the manual instructions. After staining, cells were re-suspended in RPMI-1640 complete medium containing 10% FBS, and were added into pre-coated 96-well plate. When performing IFN-γ secretion test inhibited by Vδ1 T cells, both Vδ1 T cells and CD4 T cells were added into pre-coated 96-well plates at 1: 2 ratio. After co-incubation for 72 h, 100X PMA+Ion was added. After 6-h continuous incubation at 37°C, cells were collected for assaying IFN-γ secretion level.

IL-10 secretion assay by Vδ1 T cells

Vδ1 T cells cultured in 96-well plates to which 100X PMA+Ion was added, followed by 6-h continuous incubation at 37°C. Cells were collected and added to 0.5 mL membrane fracture buffer for 30-min incubation in the dark. After rinsing twice in lysis buffer, APC-IL-10 antibody was added for incubation in the dark at room temperature for 30 min. Cells were finally rinsed twice in lysis buffer and re-suspended in 0.1 mL PBS.

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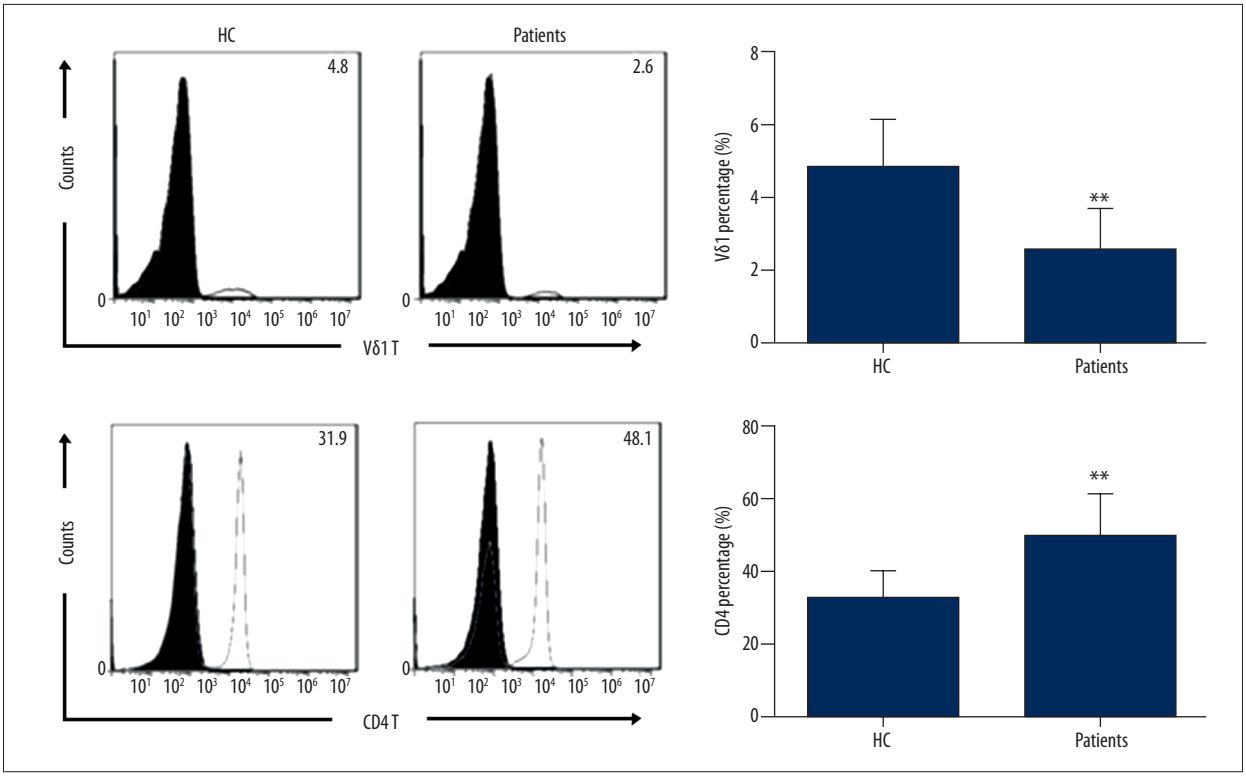

Figure 1. Vδ1 T cell/CD4 T cell ratio in AS patients by flow cytometry. ** p<0.01 compared to healthy control (HC) group.

Statistical analysis

SPSS 16.0 software was used to analyze all collected data. Between-group comparison was performed using the *t* test, while multiple-group comparison was done using analysis of variance (ANOVA). A statistically significant difference was defined as p<0.05.

Results

Vδ1 T cell/CD4 T cell ratio

As shown in Figure 1, Vδ1 T cell percentage in healthy PBMCs was (4.81±1.33)% and was decreased to (2.54±1.12)% in AS patients. As compared to control individuals, the ratio of Vδ1 T cells in AS patients was significantly decreased (p<0.01). The ratio of CD4 T cells was (32.1±8.04)% and (49.8±11.63)% in control and AS patients, respectively. Compared to controls, AS patients had significantly higher CD4 T cell ratios (p<0.01).

Vδ1 T cell-directed immune suppression in AS patients

As shown in Figure 2, the proliferation activity of naïve CD4 T cells in healthy PBMCs was (85.1±10.92)%, and was (60.4±8.96)% after co-incubation with Vδ1 T cells. In PBMCs of AS patients, the proliferation ability of naïve CD4 T cells in healthy PBMCs was (83.1±11.38)%, and was (26.7±6.84)% after co-incubation with Vδ1 T cells. Compared to the control group, AS patients had significantly depressed immune suppression function by peripheral Vδ1 T cells (p<0.01).

Inhibition of IFN-γ secretion of CD4 T cells by peripheral Vδ1 T cells

As shown in Figure 3, the percentage of IFN-γ+ CD4 T cells in healthy controls was (36.3±7.31)% when incubated alone and (18.3±5.13)% when co-incubated with Vδ1 T cells. The ratio of IFN-γ+ CD4 T cells in AS patients, however, was (35.9±7.24)% when incubated alone and (26.9±5.42)% when co-incubated with Vδ1 T cells. Compared to the healthy control group, peripheral blood Vδ1 T cells in AS patients had significantly depressed inhibitory function on CD4 T cells for secreting IFN-γ (p<0.01).

IL-10 secretion level by peripheral Vδ1 T cells

As shown in Figure 4, the percentage of IL-10+ Vδ1 T cells was (8.13±2.35)% and (4.02±1.14)% in PBMCs of healthy controls and AS patients, respectively. Compared to controls, AS patients had significantly depressed IL-10 secretion level by PBMC Vδ1 T cells (p<0.01).

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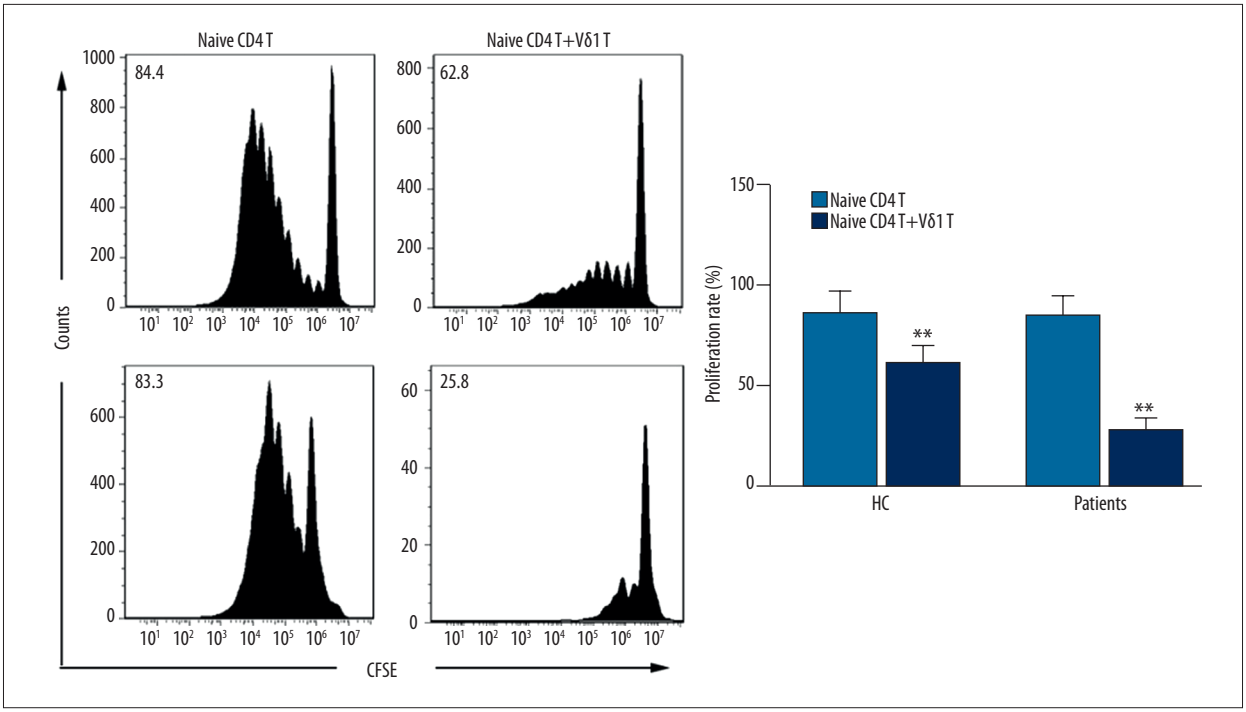

Figure 2. Vδ1 T cell-directed immune suppression assay. ** p<0.01 compared to healthy control (HC) group.

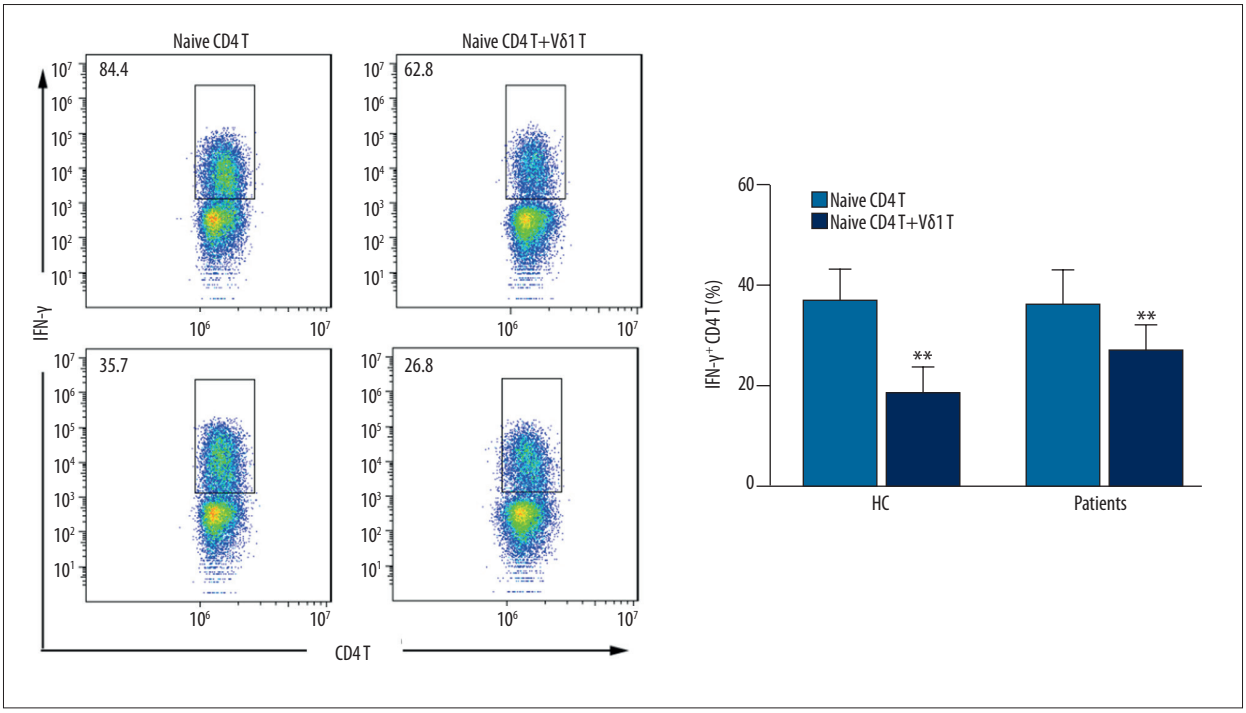

Figure 3. Inhibition of IFN-γ secretion from CD4 T cells by Vδ1 T cells. ** p<0.01 compared to healthy control (HC) group.

Discussion

AS is an autoimmune disease involving chronic inflammation of the sacroiliac joint and spine. It has an incidence at ~0.3% in China, and is a major cause of disability [1–6]. The pathogenesis of AS involves multiple factors, but the precise cause is unknown. Regulatory T cells (Treg) are lymphocytes with immune-suppressing functions, and play important roles in maintaining body immune homeostasis [13–15]. Studies have revealed the close relationship between imbalance of body

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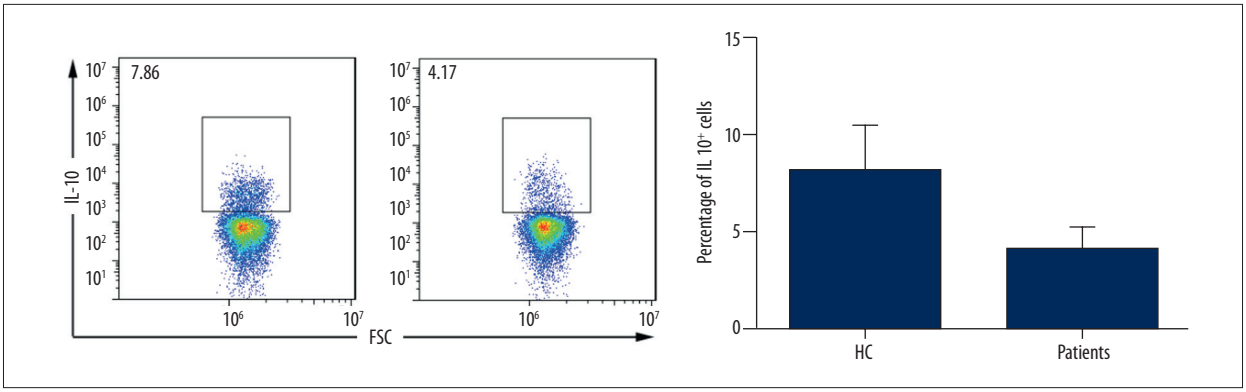

Figure 4. IL-10 secretion by Vδ1 T cells. ** p<0.01 compared to healthy control (HC) group.

immune cells and the pathogenesis/progression of AS [7,8]. Anti-TNF-α treats AS via downregulating the peripheral Th17 cell ratio and related cytokines, and up-regulating Treg cell percentage in peripheral blood [8], suggesting the critical role of Treg in the pathogenesis of AS.

Other immune regulatory cells besides CD4 Treg also exist in peripheral blood, such as regulatory B cells (Breg) and Vδ1 T cells [9-11]. Vδ1 T cells are a kind of T cell recently demonstrated to have immune-suppressing functions [11]. Little is known about the expression and function of Vδ1 T cells in peripheral blood of AS patients. Therefore, this study observed the ratio of peripheral Vδ1 T cells and their functions in AS patients, and discussed the role of Vδ1 T cells in AS pathogenesis. We found significantly lowered Vδ1 T cells ratio in AS patients compared to controls (p<0.01). We also found significantly elevated peripheral CD4 T cells in AS patients compared to controls (p<0.01). These results were consistent with previous reports [16-18].

The predominant phenotype of Breg is CD19(+)CD24(hi) CD38(hi) B cells, which have a dual function in inhibiting IFN-γ and TNF-α in CD4 T cells. This *in vitro* study confirmed the inhibitory role of peripheral Vδ1 T cells in inhibiting IFN-γ secretion from CD4 T cells in healthy controls and AS patients. However, this inhibitory role in AS patients was significantly lower in AS patients compared to controls (p<0.01). Results of the functional assay suggest the significantly lowered inhibitory ability of Vδ1 T cells in AS patients on proliferation of naïve CD4 T cells. Combined with the elevation of peripheral CD4 T cells in AS patients, it is proposed that the weakening of immune suppression potency of peripheral Vδ1 T cells leads to the elevation of peripheral CD4 T cells.

Previous studies have reported that Breg cells mainly exert immune-suppressing functions via secreting IL-10. CD19(+) CD24(hi)CD38(hi) B cell ratio in hypercholesterolemia mice was increased, and can inhibit disease progression via IL-10 [19]. Similar to Breg, Vδ1 T cells also mainly exert their immune-suppressing functions via IL-10. In RA patients, the ratio of Vδ1 T cells in synovial fluids was significantly elevated, suggesting the involvement of Vδ1 T cells in RA occurrence and progression [20]. This study further revealed the significantly lowered ability of Vδ1 T cells to secrete IL-10, suggesting that this abnormal function of Vδ1 T cells was mainly due to lowered IL-10 levels.

Conclusions

This study demonstrates the significantly depressed Vδ1 T cells ratio in peripheral blood of AS patients, along with dysfunction of Vδ1 T cells, which is predominantly caused by lowered IL-10 levels. This change in Vδ1 T cells may cause immune inflammation and eventual occurrence of AS.

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