Results of flow cytometric detection of γδT cells in peripheral blood of patients with ankylosing spondylitis: a pilot study

Running title: Imbalance in γδ T cell subpopulations in AS

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Abstract

Objective: Previous studies have suggested that γδT cells play an important role in the pathogenesis of ankylosing spondylitis (AS). In this pilot study, the peripheral blood mononuclear cells (PBMCs) of patients with ankylosing spondylitis (AS) and healthy volunteers were stained and analyzed by flow cytometry to distinguish γδ T cells and its subtypes, and then to report the distribution of γδ T cells and iyts subtypes and their correlation with ankylosing spondylitis.

Methods: A total of 17 patients with active AS and 10 age- and gender- matched healthy volunteers were enrolled in this study, and their peripheral blood were drawn to collect mononuclear cells (PBMCs). Flow cytometry was used to analyze γδ T cell subpopulations by measuring the surface and intracellular expressions of phenotypic markers. Serum levels of inflammatory and bone turnover markers were measured, and their correlations with subpopulations of γδ T cells were evaluated.

Results: In patients with AS, the Vδ2 fractions within γδ T cells and $CD3^+$ T cells decreased significantly, in particular, the proportions of $CD27^+$ Vδ2 T cells, $CD86^+CD80^+$ Vδ1 T cells, and IL17A-secreting and TNFα-secreting Vδ1 T cells within the parental cells decreased significantly. γδ T cells/PBMCs, Vδ2 cells/γδ T cells, and Vδ2 cells/$CD3^+$ T cells were negatively correlated with CRP, whereas Vδ1 cells/$CD3^+$ T cells were negatively correlated with ESR. Vδ1 cells/γδ T cells were positively correlated with CRP, γδ T cells/PBMCs were positively correlated with β-CTx, $CD69^+CD25^+$ and IL-17A-secreting Vδ1 cells were positively correlated with TP1NP, and $CD69^+CD25^+$ Vδ1 and Vδ2 cells were positively correlated with osteocalcin.

Conclusions: Decreases in peripheral Vδ2, $CD27^+$ Vδ2, $CD86^+CD80^+$ Vδ1, and IL17A or TNFα-secreting Vδ1 T cells are associated with AS. The correlations between γδ T cell subpopulations and CRP and the $CD69^+CD25^+$ subpopulation with TP1NP or osteocalcin suggest that an imbalance in peripheral γδ T cell subpopulations contributes to the pathogenesis of AS.

Keywords: Ankylosing spondylitis, gamma delta T cell, interleukin-17A, T-cell receptor.

Introduction

Ankylosing spondylitis (AS) is an inflammatory auto-immune disease and the most prevalent form of spondyloarthritis (SpA), with a worldwide prevalence of 7.4 to 31.9 per 10,000 individuals.(1) It is pathologically characterized by inflammation of the spine and sacroiliac joints, which results in pain, stiffness, and, eventually, new bone formation and joint ankylosis.(2) The disease has genetic susceptibility and is highly associated with HLA-B27, but, only 1% to 5% of HLA-B27-positive individuals develop AS, indicating that additional factors are also involved in the pathogenesis of AS.(3) A genome-wide association study revealed that the interleukin (IL)-23 and IL-1 cytokine pathways play crucial roles in susceptibility to AS.(3) Despite the claim by Meliconi et al. that the amount of γδ T cells remains unchanged in the peripheral blood or synovial fluid from patients with SpA,(4) Kenna et al. demonstrated that a large increase in the proportion of γδ T cells expressing the IL-23 receptor (IL-23R) is responsible for elevated IL-23R levels in the peripheral blood of patients with AS, resulting in increased IL-17 secretion and playing a pathogenic role in AS.(5) This finding emphasizes the significance of γδ T cells in the pathogenesis of AS.

Γδ T cells are a distinct T cell subpopulation that expresses the γδ T-cell receptor (TCR) instead of the αβ TCR found in the majority of T lymphocytes, and are more involved in innate immunity and homeostatic processes compared to αβ T cells.(6,7) In adult humans, the Vδ domain distinguishes two major subsets of γδ T cells. The Vδ1 subset is prevalent in the thymus and peripheral tissues and responds to antigens associated with stress. In contrast, the majority of γδ T cells in the blood are Vδ2 cells, which respond to pyrophosphate molecules.(8,9) γδ T cells produce numerous cytokines, such as

interferon-γ (IFN-γ), tumor necrosis factor α (TNF-α), IL-17, IL-21, and IL-22.(10) Vδ1 and Vδ2 subsets can both produce IL-17.(11) Recent research demonstrated that IL-17A-producing γδ T cells promote osteogenesis (12) and that anti-IL-17A therapy is effective in the treatment of AS,(13,14) indicating the significance of IL-17A-producing γδ T cells in the pathogenesis of AS, which is characterized by excessive bone formation.(15-17) In this study, we aimed to identify the subpopulations of circulating γδ T cells that may be involved in AS. We determined the proportions of various T cell subpopulations in peripheral blood mononuclear cells (PBMCs) from patients with AS and correlated them with disease activity markers. The results describe the imbalance of γδ T cell subsets in the peripheral blood of patients with AS, thereby providing new information on the pathogenesis of AS.

Materials and methods

Patients and sample collection

We enrolled 17 patients with active AS and 10 age- and sex-matched healthy controls in this study. The inclusion criteria for patients with AS were 1) a diagnosis of AS according to the modified New York criteria (18) and 2) non-treatment with biological disease-modifying anti-rheumatic drugs (DMARDs). Patients who received DMARD therapy or who had hematologic diseases, tumors, or chronic infectious diseases were excluded. Table 1 summarizes the clinical characteristics of the study participants. The Ethics Committee of Beijing Jishuitan Hospital approved this study (approval #202007-08; Beijing, China) and it was carried out in accordance with the Helsinki Declaration. Written informed consent was obtained from all participants before sample collection.

Preparation of PBMCs

A whole blood sample was collected from each study participant. PBMCs were prepared using density gradient centrifugation over Ficoll-Hypaque (GE Healthcare), as described previously.(19) In brief, whole blood diluted 50% with normal saline was added on top of the Ficoll separation medium in a ratio of 2:1, followed by centrifugation at 2000 rpm for 20 minutes at room temperature. The PBMC layer was isolated and resuspended in normal saline before centrifugation at 1,500 rpm at room temperature for 10 minutes. The PBMC pellet was resuspended in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and counted after repeated washing. The cell concentration was adjusted to 3×10^6 cells/mL.

Surface marker staining

Γδ T cells have complex phenotypes that are determined by the expression of cluster of differentiation (CD) molecules.(16) The balance between different T cell subsets and the cytokines they produce is essential for the pathogenesis of autoimmune disorders (17); however, which subsets of γδ T cells are associated with AS remains largely unknown.

CD3 can be used to determine the total T cell levels. (20) αβ T cells and γδ T cells can be subdivided from $CD3^+$ T cells. (6) We focused on γδ T cells to see if the γδ T cells are different between patients with AS and healthy controls. In addition, γδ T cells were subdivided into γδ 1 and γδ 2 subgroups, as well as a subset of immunoregulatory cells.(6) These γδ Tregs expressing Foxp3, are members of the Vδ1 subgroup, have the $CD27^+$ $CD25^{high}$ phenotype, and regulate the activity of $CD4^+$ T cells and DCs through cell-cell contact. Moreover, CD25, CD69, CD80, and CD86 are activation markers for T cell activation.(21,22) These markers were examined to determine if they were elevated in patients with AS compared to normal controls; an elevation in these markers could

indicate that γδ T cells are activated. CD4 and CD25 have regulatory and activating effects and may be associated with immune enhancement; autoimmune enhancement may indicate a more aggressive disease(23) CD80 is a co-stimulator of activated T CTLA-4, and its elevation may be associated with active immune checkpoints and disease activity.(24)

Intracellular staining of TCRs and cytokines

As the percentage of γδ T cells in peripheral blood is so low, between 3% and 5%, it is not possible to isolate sufficient cells for detection. Thus, we utilized flow cytometry intracellular cytokine staining and fluorescently labeled antibodies to various cytokines. The FITC-Vδ1 antibody (#TCR-2730) was purchased from Invitrogen (Waltham, MA, USA). PerCP/Cyanine5.5 anti-human CD3 (#300328), PE/Cyanine7 anti-human TCR Vδ2 (#331422), APC anti-human CD25 (#302610), APC/Fire™ 750 anti-human CD69 (#310946), Brilliant Violet 421™ anti-human TCR γ/δ (#331218), Brilliant Violet 510™ anti-human CD27 (#302836), Brilliant Violet 421™ anti-human CD80 (#305222), Brilliant Violet 650™ anti-human CD86 (#305428), PE anti-human TNF-α (#502909), APC anti-human IFN-γ (#502512), and Brilliant Violet 421™ anti-human IL-17A (#512322). We stained 3×10^5 PBMCs in 100 μL RPMI 1640 containing 10% FBS in the dark for 30 minutes at 4 °C with a mixture of CD3/γ/δ/Vδ2/Vδ1 antibodies or CD3/Vδ2/Vδ1/CD25/CD69/CD27/CD80/CD86 antibodies diluted in 1% bovine serum albumin (BSA).The cells were then filtered through a cell sieve after being washed with 1 mL of PBS, centrifuged at 1000 rpm for 5 minutes, and then centrifuged again. The final dilution of each antibody was 1:75. Unstained cells were used as a blank control. Multi-stained samples were detected by flow cytometry using an ACEA NovoCyte3005

flow cytometer. The strategies for gating are presented in Additional Materials 1 and 2. Each sample was categorized according to its lymphocyte group, and 15,000 cells were collected for analysis. Prior to the initial detection experiment, beads + antibody was used for compensation (half a drop of beads + antibody 1 μL).

PBMCs were seeded at a density of 3×10^6 cells/mL in a 24-well plate and stimulated for 16 hours with a cell stimulation cocktail (#00-4975-93; Invitrogen) at 37 °C in a humidified atmosphere containing 5% CO_2. Following collection and centrifugation at 1000 rpm for 5 minutes, the cells were resuspended in 1% BSA. After centrifugation at 1000 rpm for 5 minutes, the cells were resuspended in 40 μL PBS and stained with a mixture of antibodies against CD3/vδ2/vδ1/CD27 (1:20 final dilution of each antibody) for 30 minutes at 4 °C in the dark. Unstained cells were used as a blank control. The cells were then washed twice with 1% BSA and incubated with a permeabilization buffer (#2178649; eBioscience, Waltham, MA, USA) for 30 minutes at 4 °C, followed by incubation with a mixture of antibodies against TNF-α/IFN-γ/IL-17A in the dark for 30 min at 4 °C. After washing with PBS, 2×10^5 cells were collected, resuspended in PBS, and analyzed with an ACEA NovoCyte3005 flow cytometer.

Measurement of inflammation and bone turnover markers

Markers of inflammation and bone turnover were measured at the central clinical laboratory of the hospital to determine the severity of disease in patients with AS.(25) The following parameters were measured: erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), β-isomerized C-terminal telopeptides (β-CTx), procollagen type 1 amino-terminal propeptide (TP1NP), osteocalcin (OC), 25-hydroxyvitamin D3 (25(OH)VD3), and parathyroid hormone (PTH). ESR was determined utilizing the

Westergren method. All other blood biochemical markers were identified using electrochemiluminescence.(25)

Statistical analysis

SPSS 22.0 was used to conduct statistical analyses (IBM, Armonk, NY, USA). The statistical significance was determined using one-way ANOVA and the t-test for independent samples. The correlations between γδ T cell subsets and biomarkers were evaluated using the chi-squared test, Pearson's correlation analysis, Spearman's correlation analysis, and Kendall's rank correlation analysis. A P-value < 0.05 was considered statistically significant.

Results

The proportion of the Vδ2 subset in circulating γδ T cells or $CD3^+$ T cells decreased in patients with AS.

To investigate the role of different subtypes of γδ T cells in AS, we compared their abundance in peripheral blood samples from patients with AS and healthy controls using surface or intracellular markers. As shown in Table 2, there were no statistically significant differences in the proportion of Vδ1 or Vδ2 subset in total γδ T cells or total $CD3^+$ T cells between patients with AS and healthy controls based on the surface marker staining. However, the results of intracellular staining revealed that the percentage of Vδ2 subset in total γδ T cells (0.5022 ± 0.3024 vs. 0.7357 ± 0.1275; P = 0.01) or total CD3+ T cells (0.0278 (0.0144 – 0.0591) vs. 0.0674 (0.0353 – 0.1036), P = 0.027) was significantly lower in patients with AS compared to healthy controls. In contrast, the proportion of Vδ1 subset in total γδ T cells in patients with AS were remarkably higher than in healthy controls (0.4977 ± 0.3024 vs. 0.2462 ± 0.1275; P = 0.01). **Fig. 1** depicts

the representative flow cytometry plots of Vδ1 and Vδ2 composition within $CD3^+$ cells from patients with AS and healthy controls. AS may involve a decrease in peripheral Vδ2 T cells, according to these findings.

The amounts of $CD27^+$Vδ2 T cells and $CD86^+CD80^+$ Vδ1 T cells decline in patients with AS.

Next, we compared the expression of surface markers on distinct subsets of γδ T cells between patients with AS and healthy controls. Flow cytometry was utilized to distinguish between Vδ1 and Vδ2 subsets (Fig. 2A). As shown in Table 3, in the Vδ2 subset, the proportion of $CD27^+$ cells was significantly decreased in patients with AS compared to healthy controls (0.5173 ± 0.2781 vs. 0.7454 ± 0.1933; P = 0.034), whereas the proportion of $CD27^-$ cells was significantly increased (0.4767 ± 0.2750 vs. 0.2516 ± 0.189, P = 0.034). Moreover, the proportion of $CD86^+CD80^+$ Vδ1 T cells was significantly lower in patients with AS than in healthy controls (0.0004 (0–0.014) vs. 0.0133 (0.0079–0.03), P = 0.02; Fig. 2B).

IL17A-secreting and TNFα-secreting Vδ1 subsets are decreased in patients with AS.

γδ T cells produce proinflammatory cytokines that contribute to the pathophysiology of AS, such as IL-17A, TNF-α, and IFNγ.(26) As shown in Table 4 and Fig. 3, the proportions of IL17A-secreting and TNFα-secreting Vδ1 subsets in circulating γδ T cells were significantly decreased compared with healthy controls (IL17A: 0.0015 (0–0.0114) vs. 0.0105 (0.0042–0.0322)), P = 0.04; TNF-α: 0.3150 ± 0.1490 vs. 0.4393 ± 0.1180, P = 0.034).

The correlation of different γδ T cell subpopulations with the markers of disease activity.

To investigate the role of the various γδ T cell subpopulations in the development of AS, their correlations with markers of inflammation and bone turnover were analyzed. As shown in Table 5, the proportion of γδ T cells in PBMCs correlated negatively with CRP (surface: r = -0.588, P = 0.013, Fig. 4A; intracellular: r = -0.551, P = 0.022) but positively with β-CTx (intracellular: r = 0.519, P = 0.033). The proportion of Vδ1 cells in γδ T cells was positively correlated with CRP (intracellular: r = 0.544, P = 0.024), whereas the proportion of Vδ2 cells was negatively correlated with CRP (intracellular: r = -0.544, P = 0.024). These findings suggest that the decrease in peripheral Vδ2 cells may be indicative of the severity of AS. Moreover, the proportions of Vδ1 cells and Vδ2 cells in CD3+ T cells were negatively correlated with ESR (r = -0.543, P< 0.024) and CRP (r = -0.65, P < 0.005; Fig. 4B), respectively. CD69 and CD25 are activation markers for T cells.(27) $CD69^+CD25^+$ and IL17A-secreting Vδ1 cells were positively correlated with TP1NP ($CD69^+CD25^+$: r = 0.686, P < 0.005, Fig. 4C; IL17A-secreting: r = 0.565, P = 0.018, Fig. 4D). OC was positively correlated with $CD69^+CD25^+$ Vδ1 and Vδ2 cells (Fig. 4E; $CD69^+CD25^+$ V1: r = 0.689, P < 0.005; $CD69^+CD25^+$ V2: r = 0.502, P = 0.04). These findings indicate that $CD69^+CD25^+$ γδ T cells and IL17A-secreting γδ T cells contribute to the development of AS by regulating bone turnover.

Discussion

In our study, peripheral Vδ2 T cells within the γδ T or CD3+ T cell subpopulation in patients with AS were significantly decreased, whereas peripheral Vδ1 T cells within the γδ T cell subpopulation were significantly increased. These findings indicate a decreased Vδ2/Vδ1 ratio in the peripheral blood of patients with AS. Similarly, Tham et al. found a lower ratio of Vδ2/Vδ1 in the peripheral blood of pregnant patients with rheumatoid

arthritis (RA) or AS compared to healthy controls.(28) A recent study revealed that normal human entheses contain both Vδ1 and Vδ2 subsets with inducible IL-17A production independent of IL-23R. In the entheses, the proportion of V2 cells was 1.5-fold greater than the proportion of Vδ1cells, and only Vδ2 cells consistently expressed high levels of transcripts associated with the IL-23/IL-17 pathway.(29) In mice, IL-17A-producing γδ T cells increase in number and accumulate in the enthesis, aortic valve, and ciliary body in an IL-23-dependent manner.(30) Consequently, the decrease in the proportion of peripheral Vδ2 cells suggests that Vδ2 cells may migrate from peripheral blood to inflamed synovium and contribute to the occurrence and progression of AS by producing IL-17A and other inflammatory cytokines. In short, an imbalance in the γδ T cell subpopulations may contribute to the development of AS, which indicate that it may be used clinically in the future.

Previous studies showed that CD27 is widely expressed in lymphocytes, such as natural killer cells, $CD4^+$ and $CD8^+$ T cells, and primed B cells, and γδ T cells display substantial subset heterogeneity and exert complex functions ranging from T-cell assistance to antigen presentation.(16) But in this study, we found that with the exception of a significant decrease in the proportion of $CD86^+CD80^+$ Vδ1 T cells, there were no significant differences in CD69, CD25, CD80, or CD86 expression in peripheral γδ T cells in patients with AS. These findings were more difficult to explain and differ from previous studies. Ribot et al. demonstrated that production of IL-17 is restricted to $CD27^-$ γδ T cells.(31) Our data demonstrated that the proportion of $CD27^-$ cells in the Vδ2 subset was significantly higher in patients with AS than in healthy controls, suggesting that $CD27^-$ Vδ2 cells may be an important source of IL-17 in patients with AS. CD69 and

CD25 are activation markers for T cells.(27) γδ T cells have an antigen-presenting function, as indicated by the increased expressions of antigen-presenting molecules after stimulation, such as CD69, CD80, and CD86.(32) Mucosal-associated invariant T (MAIT) cells are primarily found in the gut lamina propria, and are involved in the pathogenesis of AS by producing IL-17 and TNF-α. Hayashi et al. discovered that the expression of CD69 on MAIT cells correlates with disease severity in AS.(33) Tham et al. observed that in patients with AS, the correlation between $CD69^{+}V\delta2$ cells and disease activity has a slight tendency toward statistical significance.(28) In addition, Zhao et al. demonstrated that the number of circulating $CD4^{+}CD25^{high}CD127^{low/-}$ Treg cells was lower in newly diagnosed, treatment-naive patients with AS than in healthy controls.(34) Additionally, serum levels of CD80 and CD86 are elevated in patients with AS and reflect disease severity.(35) Blocking CD86 inhibits IL-17 production by splenocytes.(36) Double-knockout of CD80 and CD-86 in mice inhibits Th17 differentiation.(37) We could only contribute these discrepancies between our findings and previous studies to the varying disease statuses and degrees of disease severity in patients, which may need a further study with a larger sample size.

The quantity of γδ T cell subpopulations may indicate the severity of AS and our study made several attempts. Previous reports indicated that inflammatory cytokines IL-17A, TNF-α-, and IFNγ are involved in the pathophysiology of AS;(26) γδ T cells express IL-1, IL-6, IL-18, IL-23, and TGFβ1 receptors to stimulate IL-17 production; γδ T cells also release additional proinflammatory cytokines, such as TNFα and IFNγ;(38) an increased proportion of peripheral Th17 cells can be observed in patients with AS compared to healthy individuals and patients with other inflammatory diseases;(39,40) γδ T cells with

IL-17A-producing and IL-23R-expressing were significantly increased in the peripheral blood of patients with AS compared to healthy controls and patients with RA.(5) In this study, however, we observed a significant decrease in peripheral Vδ1 cells secreting IL-17A or TNFα in patients with AS relative to healthy controls. Other cytokine-producing T cells exhibited no distinguishing characteristics. Th17, IL-17-producing $CD8^+$ T cells, type 3 innate lymphoid cells, and γδ T cells may produce IL-17 as a result of IL-23 stimulation.(41) In our study, the decrease in Vδ1 cells that secrete IL-17A or TNFα may be the result of negative feedback for maintaining homeostasis. What's more, CRP and ESR are common markers of systemic inflammation.(42) B-CTx originates during bone resorption and serves as a marker for bone resorption. In contrast, TP1NP and OC are produced by bone reconstruction and serve as bone formation markers.(43) We found negative correlations in this study between γδ T cells/PBMCs and CRP, Vδ2 cells/γδ T cells and CRP, Vδ2 cells/CD3+ T cells and CRP, and Vδ1 cells/CD3+ T cells and ESR. Similarly, Mo et al. found that peripheral Vδ2 T cells but not Vδ1 T cells, were significantly lower in patients with RA and negatively correlated with disease activity.(44) The Vδ2 T cells may accumulate in inflamed tissue because they produce high levels of proinflammatory cytokines, including IL-17, TNF-α, and IFN-γ. Vδ1 cells/γδ T cells and CRP, γδ T cells/PBMCs and β-CTx, $CD69^+CD25^+$ or IL17A-secreting Vδ1 cells and TP1NP, as well as $CD69^+CD25^+$ Vδ1 and Vδ2 cells and OC exhibited positive correlations, those above findings in our study may be explained as AS is characterized by new bone formation.(45) Thus, the proportions of γδ T cell subsets could be used to determine the severity of the disease in AS.

This research has several limitations as follows: First, this is a pilot study with limited our study provides only a snapshot of the subset distribution of circulating γδ T cells in patients with AS, but the changes in the γδ T cells after the treatment and the remission were not studied. What's more, due to the limited number of patients recruited, we were unable to distinguish between the γδ T subpopulations in patients with active versus in remission AS. Second, due to the scarcity of entheseal γδ T cells, there is insufficient data to study the composition of γδ T cells at the site of inflammation and to characterize the functions of γδ T cell subsets in the development of AS. It is necessary to investigate the distribution of γδ T cell subsets across blood and local inflammatory sites and to study the function of each phenotype or subset in further study with an increased sample size and a longer follow-up period. In addition, in the correlation analysis, some of the r values were between 0.5 and 0.8, suggesting that the correlation was not ideal, perhaps some of the correlations were an outcome of outliner observations.

Conclusions

In this study, we demonstrated that patients with AS had significantly fewer Vδ2 cells than healthy controls. In particular, $CD27^+$ Vδ2 T cells, $CD86^+CD80^+$ Vδ1 T cells, and $IL17A^-$ and TNFα-secreting Vδ1 T cells were reduced in patients with AS. These factors may contribute to the pathogenesis of AS. Furthermore, the fractions of γδ T cells in PBMCs, Vδ2 cells in γδ T cells, as well as Vδ1 or Vδ2 cells in $CD3^+$ T cells negatively correlated with CRP, suggesting that the imbalance in γδ T cell subpopulations may reflect the severity of the disease. We also identified a $CD69^+CD25^+$ subpopulation and observed significant positive correlations between the $CD69^+CD25^+$ subpopulation and

bone turnover markers, suggesting that $CD69^{+}CD25^{+}$ γδ T cells regulate bone turnover and contribute to the pathogenesis of AS.

Abbreviations

γδ T cell: gamma delta T cell

AS: ankylosing spondylitis

SpA: spondyloarthritis

HLA: Human leukocyte antigen

TCR: T-cell receptor

IFN-γ: interferon-γ

TNF-α: tumor necrosis factor α

CD: cluster of differentiation

PBMCs: Peripheral blood mononuclear cells

DMARD: disease-modifying anti-rheumatic drug

FBS: fetal bovine serum

CTLA: cytolytic T lymphocyte-associated antigen

BSA: bovine serum albumin

PBS: Phosphate Buffer Solution

ESR: erythrocyte sedimentation rate

CRP C-reactive protein

β-CTX: β-isomerized C-terminal telopeptides

TP1NP: procollagen type 1 amino-terminal propeptide

OC: osteocalcin

25(OH)VD3: 25-hydroxyvitamin D3

PTH: parathyroid hormone

IL: interleukin

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Beijing Jishuitan Hospital (approval #202007-08; Beijing, China). This study was conducted in accordance with the declaration of Helsinki. Written informed consent was obtained from all participants.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Availability of data and materials

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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Figure legends

Figure 1. Vδ1 and Vδ2 composition within total CD3$^+$ T cells. The peripheral blood mononuclear cells (PBMCs) of patients with active ankylosing spondylitis (AS) and healthy controls were collected. Flow cytometry was used to distinguish between Vδ1 and Vδ2 cells based on the expression of surface (CD3/Vδ2/Vδ1/CD25/CD69/CD27/CD80/CD86) or intracellular (CD3/vδ2/vδ1/CD27) markers. Flow cytometry plots of total CD3$^+$ cells from patients P3 and P14 are shown.

Figure 1

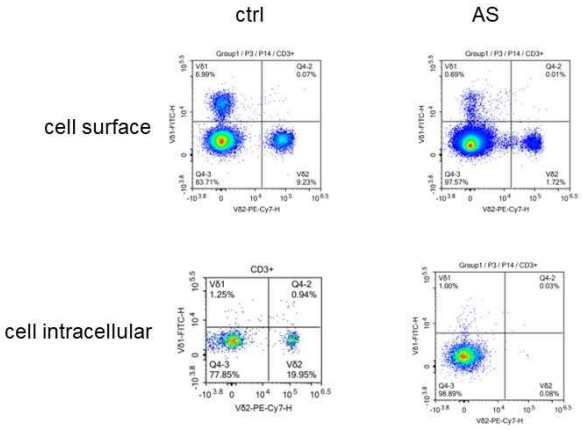

Figure 2. Analysis of CD80 and CD86 expression in Vδ1 and Vδ2 subsets. The PBMCs of patients with active AS and healthy controls were collected. Flow cytometry was used to distinguish between Vδ1 and Vδ2 cells based on surface (CD3/Vδ2/Vδ1/CD25/CD69/CD27/CD80/CD86) or intracellular (CD3/vδ2/vδ1/CD27) marker expression. (A) Using flow cytometry, the Vδ1 and Vδ2 cells were isolated from the PBMCs of two patients (P1 and P10) and healthy controls (M11). (B) The Vδ1 and Vδ2 cells were further separated based on their expression of CD80 and CD86.

Figure 2

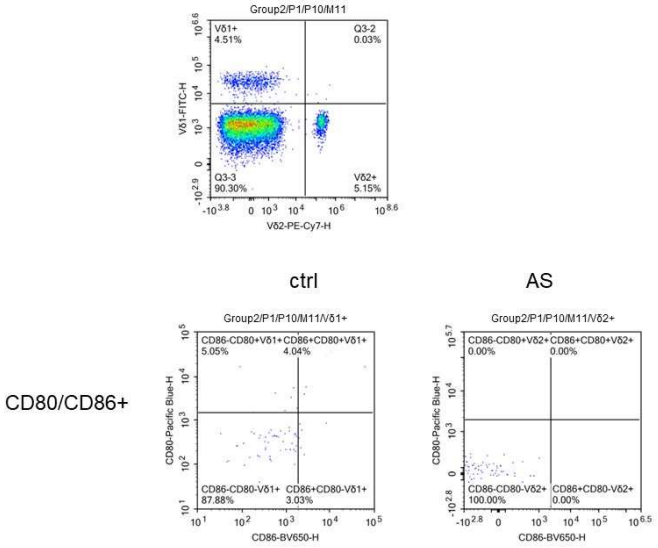

Figure 3. Flow cytometry analysis of the fractions of IL17A-secreting and TNFα-secreting Vδ1 T cells within CD3$^+$ T cells from P3 and P14. The PBMCs of patients with active AS and healthy controls were collected. Flow cytometry was used to distinguish between Vδ1 and Vδ2 cells based on surface (CD3/Vδ2/Vδ1/CD25/CD69/CD27/CD80/CD86) or intracellular (CD3/vδ2/vδ1/CD27) marker expression. Cytokine secretion was measured using antibodies against TNF-α/IFN-γ/IL-17A.

Figure 3

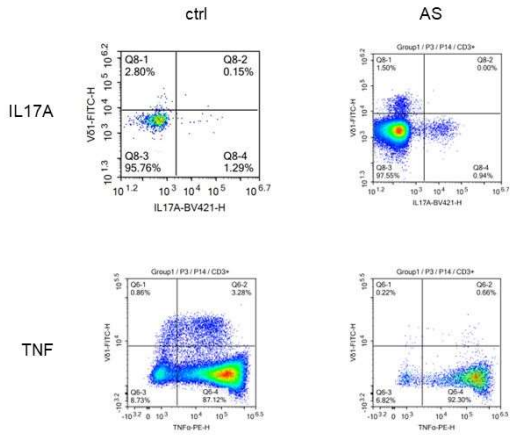

Figure 4. Correlation analysis. (A–E) Spearman's rank correlation was utilized to determine the relationship between cell subtype and inflammation or bone turnover marker expression. Each panel displays the coefficients r and corresponding *P*-values.

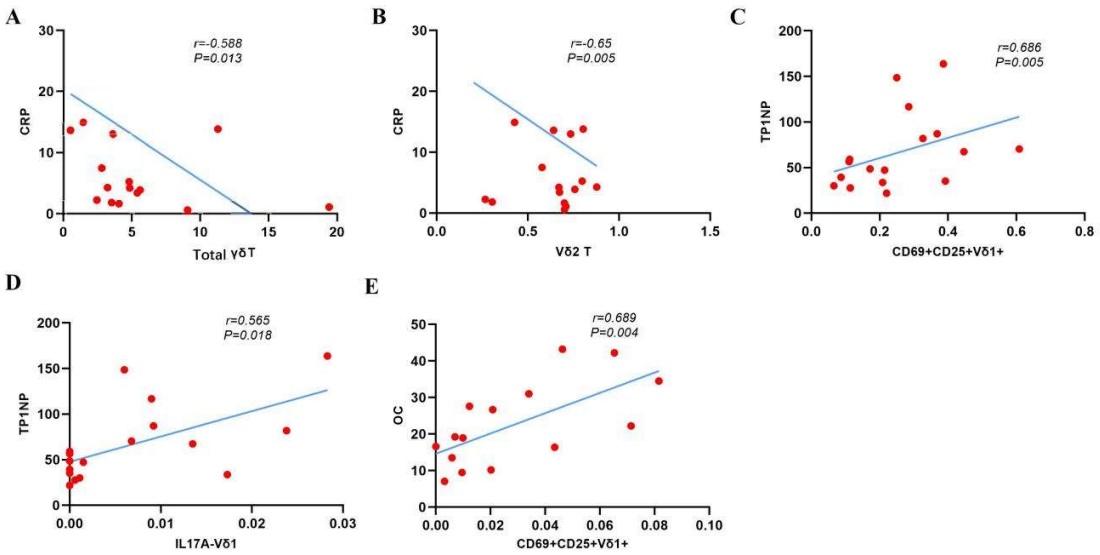

Table 1. Clinical characteristics of patients and healthy controls.

	AS patients, n = 17	Healthy controls, n = 10	P value
Male, n (%)	12 (70.6%)	4 (40.0%)	0.224
Age (years), mean±standard deviation	34.1 ± 8.7	32.1±8.3	0.567
Disease duration (years)	7.31 ± 5.24		
ASDAS-ESR, mean±standard deviation	2.75 ± 1.08		
HLA-B27 positive, n (%)	17 (100)		
ESR, mean±standard deviation	22.5 ± 15.2		
CRP, median (Q1, Q3)	4.3 (2.0 – 13.7)		
TP1NP	66.8 ± 41.7		
β-CTx, median (Q1, Q3)	0.7 (0.3 – 0.9)		
OC, mean±standard deviation	21.7 ± 11.1		
25(OH)VD3	20.9 ± 8.5		
PTH, mean±standard deviation	43.2 ± 11.3		

Note: ASDAS, Ankylosing Spondylitis Disease Activity Score; HLA-B27, human leukocyte antigen B27; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; TP1NP, procollagen type 1 amino-terminal propeptide; β-CTx, β-isomerized C-terminal telopeptides; OC, osteocalcin; 25(OH)VD3, 25-hydroxyvitamin D3; PTH, parathyroid hormone.

596 **Table 2.** Comparison of total γδ T cells and γδ T cell subsets between AS patients and healthy controls.

Staining	Subsets	AS (n = 17)	Healthy controls (n = 10)	P value
Surface staining				
	Total γδ T cells/PBMCs	0.0362 (0.0222 – 0.0551)	0.0776 (0.037 – 0.1226)	0.063
	Vδ1 T cells/total γδ T cells	0.4272 (0.1673 – 0.7088)	0.3936 (0.1809 – 0.4403)	0.482
	Vδ2 T cells/total γδ T cells	0.5728 (0.2912 – 0.8327)	0.6064 (0.5597 – 0.8191)	0.482
	Vδ1 T cells/$CD3^+$ T cells	0.0096 (0.0066 – 0.0188)	0.0194 (0.0111 – 0.0528)	0.095
	Vδ2 T cells/$CD3^+$ T cells	0.0232 (0.0062 – 0.0351)	0.0424 (0.0252 – 0.0887)	0.059
Intracellular staining				
	Total Vδ Tcells/PBMCs	0.0583 (0.0337 – 0.1225)	0.0935 (0.0548 – 0.1544)	0.269
	Vδ1 T cells/total γδ T cells	0.4977 ± 0.3024	0.2462 ± 0.1275	0.01
	Vδ2 T cells/total γδ T cells	0.5022 ± 0.3024	0.7357 ± 0.1275	0.01
	Vδ1 T cells/$CD3^+$ T cells	0.02 (0.0125 – 0.029)	0.0213 (0.0099 – 0.0363)	>0.999
	Vδ2 T cells/$CD3^+$ T cells	0.0278 (0.0144 – 0.0591)	0.0674 (0.0353 – 0.1036)	0.027

597 Note: AS, ankylosing spondylitis; PBMC, peripheral blood mononuclear cells.

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600 **Table 3.** Comparison of marker expression on γδ T cells between AS patients and healthy controls.

Subsets	AS (n = 17)	Healthy controls (n = 10)	P value
$CD27^+$ γδ T cells/Vδ1 T cells	0.567 ± 0.258	0.5310 ± 0.1848	0.703
$CD27^-$ γδ T cells/Vδ1 T cells	0.2999 ± 0.1814	0.4329 ± 0.1900	0.091
$CD27^+$ γδ T cells/Vδ2 T	0.5173 ± 0.2781	0.7454 ± 0.1933	0.034
$CD27^-$ γδ T cells/Vδ2 T cells	0.4767 ± 0.2750	0.2516 ± 0.1895	0.034
$CD69^-CD25^+$Vδ1 T cells/Parent	0.0321 (0.0185 – 0.044)	0.0157 (0.0091 – 0.0419)	0.209
$CD69^-CD25^-$Vδ1+ % Parent	0.7252 (0.5204 – 0.8507)	0.7104 (0.4844 – 0.7955)	0.912
$CD69^-CD25^+$Vδ2+ % Parent	0.0073 (0.0018 – 0.0353)	0.0071 (0.0029 – 0.0434)	0.407
$CD69^-CD25^-$Vδ2+ % Parent	0.6502 (0.2 – 0.9175)	0.8653 (0.6154 – 0.8868)	0.471
$CD86^-CD80^+$Vδ1+ % Parent	0.0318 (0.0011 – 0.0653)	0.0434 (0.0313 – 0.1289)	0.115
$CD86^-CD80^+$Vδ2+ % Parent	0.0003 (0 – 0.0018)	0 (0 – 0.0009)	0.856
$CD86^-CD80^-$Vδ1+ % Parent	0.9374 (0.8253 – 0.9589)	0.8599 (0.7921 – 0.9153)	0.155
$CD86^-CD80^-$Vδ2+ % Parent	0.9893 ± 0.0123	0.9841 ± 0.0084	0.369
$CD69^+CD25^-$Vδ1+ % Parent	0.2606 ± 0.1461	0.2785 ± 0.1617	0.727
$CD69^+CD25^-$Vδ2+ % Parent	0.275 (0.0756 – 0.72）	0.1175 (0.0959 – 0.3537)	0.802

Subsets	AS (n = 17)	Healthy controls (n = 10)	P value
$CD69^{+}CD25^{+}V\delta1+$ % Parent	0.0202 (0.007 – 0.0463)	0.0081 (0.002 – 0.0523)	0.292
$CD69^{+}CD25^{+}V\delta2+$ % Parent	0.0071 (0 – 0.0489）	0.0017 (0 – 0.0155)	0.283
$CD86^{+}CD80^{-}V\delta1+$ % Parent	0.0203 (0.0077 – 0.0924)	0.0454 (0.0242 – 0.1089)	0.156
$CD86^{+}CD80^{-}V\delta2+$ % Parent	0.0068 ± 0.0103	0.0155 ± 0.0087	0.092
$CD86^{+}CD80^{+}V\delta1+$ % Parent	0.0004 (0 – 0.014)	0.0133 (0.0079 – 0.03)	0.02
$CD86^{+}CD80^{+}V\delta2+$ % Parent	0 (0 – 0.001)	0 (0 – 0)	0.064

Note: AS, ankylosing spondylitis.

611 **Table 4.** Comparison of cytokine secretion of γδ T cells between AS patients and healthy controls.

Subsets	AS (n = 17)	Healthy controls (n = 10)	P value
IFNγ-secreting Vδ1 cells/Parent	0.4941 ± 0.2634	0.4808 ± 0.1851	0.889
IFNγ-secreting Vδ2 cells/Parent	0.5565 (0.3702 – 0.8757)	0.9242 (0.7034 – 0.9707)	0.056
IL17A-secreting Vδ1 cells/Parent	0.0015 (0 – 0.0114)	0.0105 (0.0042 – 0.0322)	0.04
IL17A- secreting Vδ2 cells/Parent	0 (0 – 0.0019)	0.0003 (0 – 0.0029)	0.44
TNFα- secreting Vδ1 cells/Parent	0.3150 ± 0.1490	0.4393 ± 0.1180	0.034
TNFα- secreting Vδ2 cells/Parent	0.3082 ± 0.1932	0.3751 ± 0.0875	0.231

612 Note: AS, ankylosing spondylitis.

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621 **Table 5.** Correlation of γδ T cell subsets with inflammation and bone turnover markers in AS patients.

Subsets	CRP		ESR		TP1NP		β-CTx		OC		25(OH)VD3		*PTH*	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
γδ T cells/PBMC (surface)	-0.588	0.013	0.015	0.955	0.086	0.743	0.291	0.258	0.105	0.687	-0.083	0.75	-0.083	0.75
Vδ1 T cells/$CD3^+$ T cells (surface)	-0.387	0.125	-0.07	0.79	-0.306	0.232	0.021	0.937	-0.228	0.379	0.021	0.937	-0.283	0.271
Vδ1 T cells/γδ T cells (surface)	0.306	0.232	-0.036	0.892	-0.321	0.209	-0.294	0.252	-0.213	0.411	0.175	0.501	-0.071	0.786
Vδ2 T cells/ $CD3^+$ T cells (surface)	-0.463	0.061	0.099	0.704	0.284	0.269	0.414	0.098	0.245	0.343	-0.123	0.639	-0.031	0.907
Vδ2 T cells/γδ T cells (surface)	-0.306	0.232	0.036	0.892	0.321	0.209	0.294	0.252	0.213	0.411	-0.175	0.501	0.071	0.786
$CD27^+$ γδ T cells/Vδ1 T cells	0.136	0.63	0.13	0.643	0.504	0.056	0.318	0.248	0.414	0.125	-0.265	0.341	0.241	0.386
$CD27^-$ γδ T cells/Vδ1 T cells	-0.046	0.869	0.109	0.699	-0.289	0.296	-0.098	0.727	-0.057	0.84	0.306	0.268	0.018	0.95
$CD27^+$ γδ T cells/Vδ2 T cells	0.043	0.879	0.063	0.825	0.204	0.467	0.364	0.182	0.2	0.475	-0.189	0.499	0.399	0.141
$CD27^-$ γδ T cells/Vδ2 T cells	-0.046	0.869	-0.084	0.766	-0.182	0.516	-0.021	0.94	-0.182	0.516	0.213	0.447	-0.395	0.145
$CD69^-CD25^+V\delta1^+$	-0.397	0.115	0.11	0.673	0.147	0.573	0.235	0.363	0.235	0.363	-0.059	0.822	0.071	0.786
$CD69^-CD25^+V\delta2^+$	0.156	0.549	-0.033	0.901	0.507	0.038	0.206	0.429	0.378	0.135	-0.281	0.274	0.308	0.23
$CD69^-CD25^-V\delta1^+$	-0.114	0.685	0.025	0.929	-0.607	0.016	-0.286	0.301	-0.364	0.182	0.352	0.198	-0.27	0.331

Subsets	CRP		ESR		TP1NP		β-CTx		OC		25(OH)VD3		*PTH*	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
$CD69^{-}CD25^{-}V\delta2^{+}$	0.082	0.771	-0.002	0.995	-0.314	0.254	-0.011	0.97	-0.257	0.355	0.411	0.128	-0.443	0.098
$CD86^{-}CD80^{+}V\delta1^{+}$	-0.119	0.779	0.119	0.779	-0.452	0.26	-0.31	0.456	-0.452	0.26	0.095	0.823	0.095	0.823
$CD86^{-}CD80^{+}V\delta2^{+}$	-0.382	0.351	-0.136	0.747	-0.136	0.747	-0.136	0.747	-0.027	0.949	0.546	0.162	0.409	0.314
$CD86^{-}CD80^{-}V\delta1^{+}$	0.314	0.544	-0.371	0.468	0.2	0.704	0.086	0.872	0.2	0.704	0.257	0.623	0.314	0.544
$CD86^{-}CD80^{-}V\delta2^{+}$	-0.029	0.957	-0.203	0.7	-0.058	0.913	-0.029	0.957	-0.232	0.658	-0.261	0.618	-0.377	0.461
$CD69^{+}CD25^{-}V\delta1^{+}$	0.12	0.646	-0.08	0.761	0.52	0.033	0.411	0.101	0.37	0.144	-0.395	0.117	0.151	0.563
$CD69^{+}CD25^{-}V\delta2^{+}$	-0.172	0.51	-0.258	0.318	0.355	0.162	0.102	0.698	0.279	0.277	-0.363	0.152	0.314	0.22
$CD69^{+}CD25^{+}V\delta1^{+}$	0.386	0.156	0.42	0.119	0.686	0.005	0.391	0.149	0.689	0.004	-0.134	0.634	0.379	0.164
$CD69^{+}CD25^{+}V\delta2^{+}$	0.187	0.471	0.047	0.859	0.402	0.109	0.199	0.444	0.502	0.04	-0.272	0.291	0.298	0.246
$CD86^{+}CD80^{-}V\delta1^{+}$	-0.238	0.57	0.163	0.699	0.143	0.736	0.048	0.911	0.071	0.867	-0.071	0.867	-0.143	0.736
$CD86^{+}CD80^{-}V\delta2^{+}$	-0.266	0.524	-0.241	0.565	0.216	0.608	0.089	0.834	0.254	0.544	0.393	0.335	0.203	0.63
$CD86^{+}CD80^{+}V\delta1^{+}$	-0.342	0.406	-0.368	0.37	0.393	0.335	0.114	0.788	0.152	0.719	-0.19	0.652	-0.203	0.63
$CD86^{+}CD80^{+}V\delta2^{+}$	-0.027	0.949	-0.382	0.351	0.627	0.096	0.355	0.389	0.546	0.162	0.027	0.949	-0.082	0.847
γδ T cells/PBMCs (intracellular)	-0.551	0.022	-0.205	0.43	0.412	0.101	0.519	0.033	0.324	0.205	0.093	0.722	0.102	0.698

Subsets	CRP		ESR		TP1NP		β-CTx		OC		25(OH)VD3		*PTH*	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
Vδ1 T cells/$CD3^+$ T cells (intracellular)	-0.262	0.309	-0.543	0.024	0.39	0.122	0.286	0.266	0.115	0.66	-0.047	0.859	0.115	0.66
Vδ1 T cells/ γδ T cells (intracellular)	0.544	0.024	-0.061	0.815	0.135	0.606	-0.158	0.544	0.154	0.554	0.086	0.743	0.124	0.636
Vδ2 T cells/$CD3^+$ T cells (intracellular)	-0.65	0.005	-0.142	0.586	0.199	0.445	0.435	0.081	0.105	0.687	-0.108	0.68	-0.052	0.844
Vδ2 T cells/ γδ T cells (intracellular)	-0.544	0.024	0.061	0.815	-0.135	0.606	0.158	0.544	-0.154	0.554	-0.086	0.743	-0.124	0.636
IFNγ-Vδ1	-0.13	0.619	0.075	0.775	0.255	0.323	0.406	0.106	0.439	0.078	0.416	0.097	-0.016	0.952
IFNγ-Vδ2	-0.137	0.599	0.098	0.708	-0.463	0.061	-0.199	0.445	-0.377	0.135	0.12	0.646	-0.265	0.304
IL17A-Vδ1	-0.054	0.837	0.085	0.747	0.565	0.018	0.319	0.212	0.46	0.063	-0.108	0.68	0.253	0.328
IL17A-Vδ2	-0.295	0.25	-0.089	0.734	-0.123	0.637	-0.011	0.965	-0.029	0.913	0.158	0.545	0.3	0.242
TNFα-Vδ1	-0.199	0.445	0.229	0.376	-0.199	0.445	-0.038	0.885	-0.172	0.51	0.022	0.933	-0.172	0.51
TNFα-Vδ2	0.005	0.985	0.327	0.2	-0.385	0.127	-0.395	0.117	-0.456	0.066	-0.282	0.273	-0.206	0.428

Note: r, coESR, erythrocyte sedimentation rate; CRP, C-reactive protein; β-CTx, β-isomerized C-terminal telopeptides; TP1NP, procollagen type 1 amino-terminal propeptide; OC, osteocalcin; 25(OH)VD3, 25-hydroxyvitamin D3; PTH, parathyroid hormone.