

# Results of Flow Cytometric Detection of $\gamma\delta$ T Cells in Peripheral Blood of Patients With Ankylosing Spondylitis: A Pilot Study

Si-Liang MAN<sup>1#</sup>, Peng DONG<sup>1,2#</sup>, Wei LIU<sup>1</sup>, Hong-Chao LI<sup>1</sup>, Liang ZHANG<sup>3</sup>, Xiao-Jian JI<sup>4</sup>, Li-Dong HU<sup>4</sup>, Hui SONG<sup>1</sup>

<sup>#</sup>These authors contributed equally to this work

<sup>1</sup>Department of Rheumatology, Beijing Jishuitan Hospital, Capital Medical University, Beijing, China, <sup>2</sup>Changzhou Xitaihu Institute for Frontier Technology of Cell Therapy, Wujin District, Changzhou, China, <sup>3</sup>Department of Orthopedic Surgery, Beijing Jishuitan Hospital, Capital Medical University, Beijing, China, <sup>4</sup>Department of Rheumatology, Chinese PLA General Hospital, Beijing, China

Received May 24, 2023

Accepted August 30, 2023

## Summary

Previous studies have suggested that  $\gamma\delta$ T 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  $\gamma\delta$ T cells and its subtypes, and then to report the distribution of  $\gamma\delta$ T cells and its subtypes and their correlation with ankylosing spondylitis. 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  $\gamma\delta$ 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  $\gamma\delta$ T cells were evaluated. In patients with AS, the  $V\delta 2$  fractions within  $\gamma\delta$ T cells and  $CD3^+$ T cells decreased significantly, in particular, the proportions of  $CD27^+V\delta 2$ T cells,  $CD86^+CD80^+V\delta 1$ T cells, and IL17A-secreting and TNF $\alpha$ -secreting  $V\delta 1$ T cells within the parental cells decreased significantly.  $\gamma\delta$ T cells/PBMCs,  $V\delta 2$  cells/ $\gamma\delta$ T cells, and  $V\delta 2$  cells/ $CD3^+$ T cells were negatively correlated with CRP, whereas  $V\delta 1$  cells/ $CD3^+$ T cells were negatively correlated with ESR.  $V\delta 1$  cells/ $\gamma\delta$ T cells were positively correlated with CRP,  $\gamma\delta$ T cells/PBMCs were positively correlated with  $\beta$ -CTX,  $CD69^+CD25^+$  and IL-17A-secreting  $V\delta 1$  cells were positively correlated with TP1NP, and  $CD69^+CD25^+V\delta 1$  and  $V\delta 2$  cells were positively correlated with osteocalcin. Decreases in peripheral  $V\delta 2$ ,  $CD27^+V\delta 2$ ,  $CD86^+CD80^+V\delta 1$ , and IL17A or TNF $\alpha$ -

secreting  $V\delta 1$ T cells are associated with AS. The correlations between  $\gamma\delta$ T cell subpopulations and CRP and the  $CD69^+CD25^+$  subpopulation with TP1NP or osteocalcin suggest that an imbalance in peripheral  $\gamma\delta$ T cell subpopulations contributes to the pathogenesis of AS.

## Keywords

Ankylosing spondylitis • Gamma delta T cell • Interleukin-17A • T-cell receptor

## Corresponding author

Hui Song, Department of Rheumatology, Beijing Jishuitan Hospital, Capital Medical University, No. 31 Xijiekou East Street, Xicheng District Beijing 100035, China. Email: [jst\\_fsmyk@126.com](mailto:jst_fsmyk@126.com)

## 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 ankyloses [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  $\gamma\delta$  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  $\gamma\delta$  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  $\gamma\delta$  T cells in the pathogenesis of AS.

$\Gamma\delta$  T cells are a distinct T cell subpopulation that expresses the  $\gamma\delta$  T-cell receptor (TCR) instead of the  $\alpha\beta$  TCR found in the majority of T lymphocytes, and are more involved in innate immunity and homeostatic processes compared to  $\alpha\beta$  T cells [6,7]. In adult humans, the V $\delta$  domain distinguishes two major subsets of  $\gamma\delta$  T cells. The V $\delta$ 1 subset is prevalent in the thymus and peripheral tissues and responds to antigens associated with stress. In contrast, the majority of  $\gamma\delta$  T cells in the blood are V $\delta$ 2 cells, which respond to pyrophosphate molecules [8,9].  $\gamma\delta$  T cells produce numerous cytokines, such as interferon- $\gamma$  (IFN- $\gamma$ ), tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), IL-17, IL-21, and IL-22 [10]. V $\delta$ 1 and V $\delta$ 2 subsets can both produce IL-17 [11]. Recent research demonstrated that IL-17A-producing  $\gamma\delta$  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

$\gamma\delta$  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  $\gamma\delta$  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  $\gamma\delta$  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.

**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		
$\beta$ -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

### 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  $3 \times 10^6$  cells/mL.

#### *Surface marker staining*

$\Gamma\delta$  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  $\gamma\delta$  T cells are associated with AS remains largely unknown.

CD3 can be used to determine the total T cell levels. [20].  $\alpha\beta$  T cells and  $\gamma\delta$  T cells can be subdivided from CD3<sup>+</sup> T cells [6]. We focused on  $\gamma\delta$  T cells to see if the  $\gamma\delta$  T cells are different between patients with AS and healthy controls. In addition,  $\gamma\delta$  T cells were subdivided into  $\gamma\delta$  1 and  $\gamma\delta$  2 subgroups, as well as a subset of immunoregulatory cells [6]. These  $\gamma\delta$  Tregs expressing Foxp3, are members of the V $\delta$ 1 subgroup, have the CD27<sup>+</sup> CD25<sup>high</sup> phenotype, and regulate the activity of CD4<sup>+</sup> 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  $\gamma\delta$  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  $\gamma\delta$  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-V $\delta$ 1 antibody (#TCR-2730) was purchased from Invitrogen (Waltham, MA, USA). PerCP/Cyanine5.5 anti-human CD3 (#300328), PE/Cyanine7 anti-human TCR V $\delta$ 2

(#331422), APC anti-human CD25 (#302610), APC/Fire™ 750 anti-human CD69 (#310946), Brilliant Violet 421™ anti-human TCR  $\gamma/\delta$  (#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- $\alpha$  (#502909), APC anti-human IFN- $\gamma$  (#502512), and Brilliant Violet 421™ anti-human IL-17A (#512322). We stained  $3 \times 10^5$  PBMCs in 100  $\mu$ L RPMI 1640 containing 10 % FBS in the dark for 30 minutes at 4 °C with a mixture of CD3/ $\gamma/\delta$ /V $\delta$ 2/V $\delta$ 1 antibodies or CD3 / V $\delta$ 2 / V $\delta$ 1 / 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 [Supplementary Materials 1](#) and [Supplementary Materials 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  $\mu$ L).

PBMCs were seeded at a density of  $3 \times 10^6$  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<sub>2</sub>. 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  $\mu$ L PBS and stained with a mixture of antibodies against CD3/ $\gamma/\delta$ 2/ $\gamma/\delta$ 1/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- $\alpha$ /IFN- $\gamma$ /IL-17A in the dark for 30 min at 4 °C. After washing with PBS,  $2 \times 10^5$  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),  $\beta$ -isomerized C-terminal telopeptides ( $\beta$ -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  $\gamma\delta$  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 V $\delta$ 2 subset in circulating $\gamma\delta$ T cells or CD3+ T cells decreased in patients with AS

To investigate the role of different subtypes of  $\gamma\delta$  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 V $\delta$ 1 or V $\delta$ 2 subset in total  $\gamma\delta$  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 V $\delta$ 2 subset in total  $\gamma\delta$  T cells ( $0.5022 \pm 0.3024$  vs.  $0.7357 \pm 0.1275$ ;  $P=0.01$ ) 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 V $\delta$ 1 subset in total  $\gamma\delta$  T cells in patients with AS were remarkably higher than in healthy controls ( $0.4977 \pm 0.3024$  vs.  $0.2462 \pm 0.1275$ ;  $P=0.01$ ). Fig. 1 depicts the representative flow cytometry plots of V $\delta$ 1 and V $\delta$ 2 composition within CD3+ cells from patients with AS and healthy controls. AS may involve a decrease in peripheral V $\delta$ 2 T cells, according to these findings.

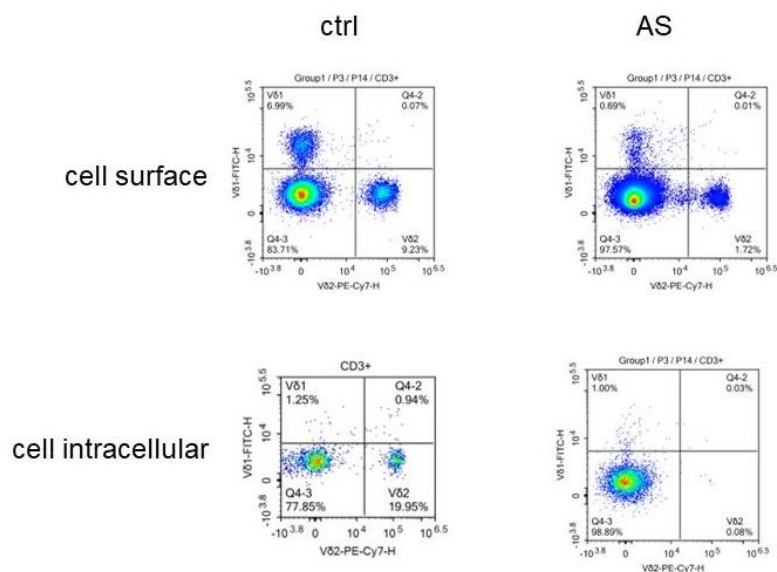
#### The amounts of CD27+V $\delta$ 2 T cells and CD86+CD80+V $\delta$ 1 T cells decline in patients with AS

Next, we compared the expression of surface markers on distinct subsets of  $\gamma\delta$  T cells between patients with AS and healthy controls. Flow cytometry was utilized to distinguish between V $\delta$ 1 and V $\delta$ 2 subsets (Fig. 2A).

**Table 2.** Comparison of total  $\gamma\delta$  T cells and  $\gamma\delta$  T cell subsets between AS patients and healthy controls.

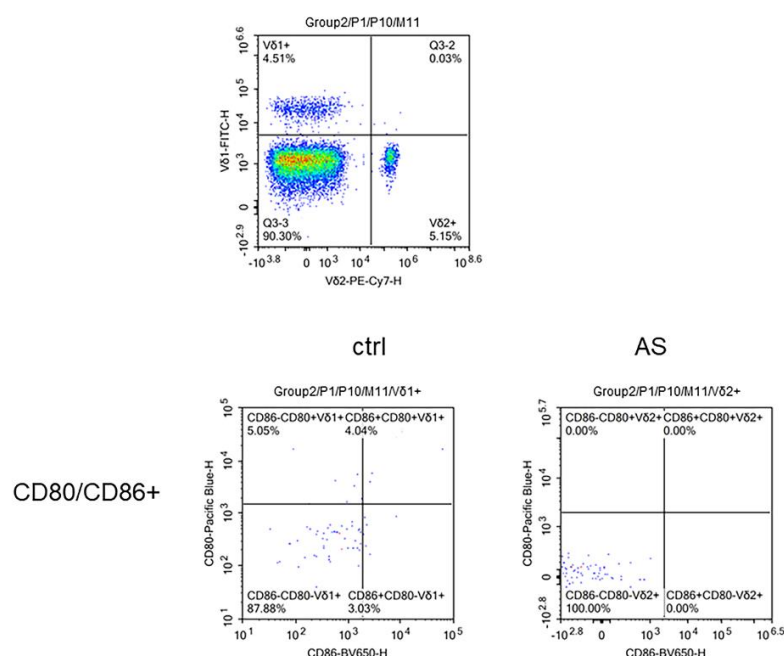
Staining	Subsets	AS (n = 17)	Healthy controls (n = 10)	P value
<i>Surface staining</i>				
	Total $\gamma\delta$ T cells/PBMCs	0.0362 (0.0222 – 0.0551)	0.0776 (0.037 – 0.1226)	0.063
	V $\delta$ 1 T cells/total $\gamma\delta$ T cells	0.4272 (0.1673 – 0.7088)	0.3936 (0.1809 – 0.4403)	0.482
	V $\delta$ 2 T cells/total $\gamma\delta$ T cells	0.5728 (0.2912 – 0.8327)	0.6064 (0.5597 – 0.8191)	0.482
	V $\delta$ 1 T cells/CD3+ T cells	0.0096 (0.0066 – 0.0188)	0.0194 (0.0111 – 0.0528)	0.095
	V $\delta$ 2 T cells/CD3+ T cells	0.0232 (0.0062 – 0.0351)	0.0424 (0.0252 – 0.0887)	0.059
<i>Intracellular staining</i>				
	Total V $\delta$ T cells/PBMCs	0.0583 (0.0337 – 0.1225)	0.0935 (0.0548 – 0.1544)	0.269
	V $\delta$ 1 T cells/total $\gamma\delta$ T cells	$0.4977 \pm 0.3024$	$0.2462 \pm 0.1275$	0.01
	V $\delta$ 2 T cells/total $\gamma\delta$ T cells	$0.5022 \pm 0.3024$	$0.7357 \pm 0.1275$	0.01
	V $\delta$ 1 T cells/CD3+ T cells	0.02 (0.0125 – 0.029)	0.0213 (0.0099 – 0.0363)	0.999
	V $\delta$ 2 T cells/CD3+ T cells	0.0278 (0.0144 – 0.0591)	0.0674 (0.0353 – 0.1036)	0.027

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



**Fig. 1.** V $\delta$ 1 and V $\delta$ 2 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 V $\delta$ 1 and V $\delta$ 2 cells based on the expression of surface (CD3/V $\delta$ 2/V $\delta$ 1/CD25/CD69/CD27/CD80/CD86) or intracellular (CD3/v $\delta$ 2/v $\delta$ 1/CD27) markers. Flow cytometry plots of total CD3+ cells from patients P3 and P14 are shown.

**Fig. 2.** Analysis of CD80 and CD86 expression in V $\delta$ 1 and V $\delta$ 2 subsets. The PBMCs of patients with active AS and healthy controls were collected. Flow cytometry was used to distinguish between V $\delta$ 1 and V $\delta$ 2 cells based on surface (CD3/V $\delta$ 2/V $\delta$ 1/CD25/CD69/CD27/CD80/CD86) or intracellular (CD3/v $\delta$ 2/v $\delta$ 1/CD27) marker expression. **(A)** Using flow cytometry, the V $\delta$ 1 and V $\delta$ 2 cells were isolated from the PBMCs of two patients (P1 and P10) and healthy controls (M11). **(B)** The V $\delta$ 1 and V $\delta$ 2 cells were further separated based on their expression of CD80 and CD86.



As shown in Table 3, in the V $\delta$ 2 subset, the proportion of CD27+ cells was significantly decreased in patients with AS compared to healthy controls ( $0.5173 \pm 0.2781$  vs.  $0.7454 \pm 0.1933$ ;  $P=0.034$ ), whereas the proportion of CD27- cells was significantly increased ( $0.4767 \pm 0.2750$  vs.  $0.2516 \pm 0.189$ ,  $P=0.034$ ). Moreover, the proportion of CD86+CD80+ V $\delta$ 1 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 TNF $\alpha$ -secreting V $\delta$ 1 subsets are decreased in patients with AS*

$\gamma\delta$  T cells produce proinflammatory cytokines

that contribute to the pathophysiology of AS, such as IL-17A, TNF- $\alpha$ , and IFN $\gamma$  [26]. As shown in Table 4 and Fig. 3, the proportions of IL17A-secreting and TNF $\alpha$ -secreting V $\delta$ 1 subsets in circulating  $\gamma\delta$  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- $\alpha$ :  $0.3150 \pm 0.1490$  vs.  $0.4393 \pm 0.1180$ ,  $P=0.034$ ).

*The correlation of different  $\gamma\delta$  T cell subpopulations with the markers of disease activity.*

To investigate the role of the various  $\gamma\delta$  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  $\gamma\delta$  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  $\beta$ -CTx (intracellular:  $r=0.519$ ,  $P=0.033$ ). The proportion of V $\delta$ 1 cells in  $\gamma\delta$  T cells was positively correlated with CRP (intracellular:  $r=0.544$ ,  $P=0.024$ ), whereas the proportion of V $\delta$ 2 cells was negatively correlated with CRP (intracellular:  $r=-0.544$ ,  $P=0.024$ ). These findings suggest that the decrease in peripheral V $\delta$ 2 cells may be indicative of the severity of AS. Moreover, the proportions of V $\delta$ 1 cells and V $\delta$ 2 cells in CD3<sup>+</sup> 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<sup>+</sup>CD25<sup>+</sup> and IL17A-secreting V $\delta$ 1 cells were positively correlated with TP1NP (CD69<sup>+</sup>CD25<sup>+</sup>:  $r=0.686$ ,  $P<0.005$ , Fig. 4C; IL17A-secreting:  $r=0.565$ ,  $P=0.018$ , Fig. 4D). OC was positively correlated with CD69<sup>+</sup>CD25<sup>+</sup> V $\delta$ 1 and V $\delta$ 2 cells (Fig. 4E; CD69<sup>+</sup>CD25<sup>+</sup> V1:  $r=0.689$ ,  $P<0.005$ ; CD69<sup>+</sup>CD25<sup>+</sup> V2:  $r=0.502$ ,  $P=0.04$ ). These findings indicate that CD69<sup>+</sup>CD25<sup>+</sup>  $\gamma\delta$  T cells and IL17A-secreting  $\gamma\delta$  T cells contribute to the development of AS by regulating bone turnover.

**Table 3.** Comparison of marker expression on  $\gamma\delta$  T cells between AS patients and healthy controls.

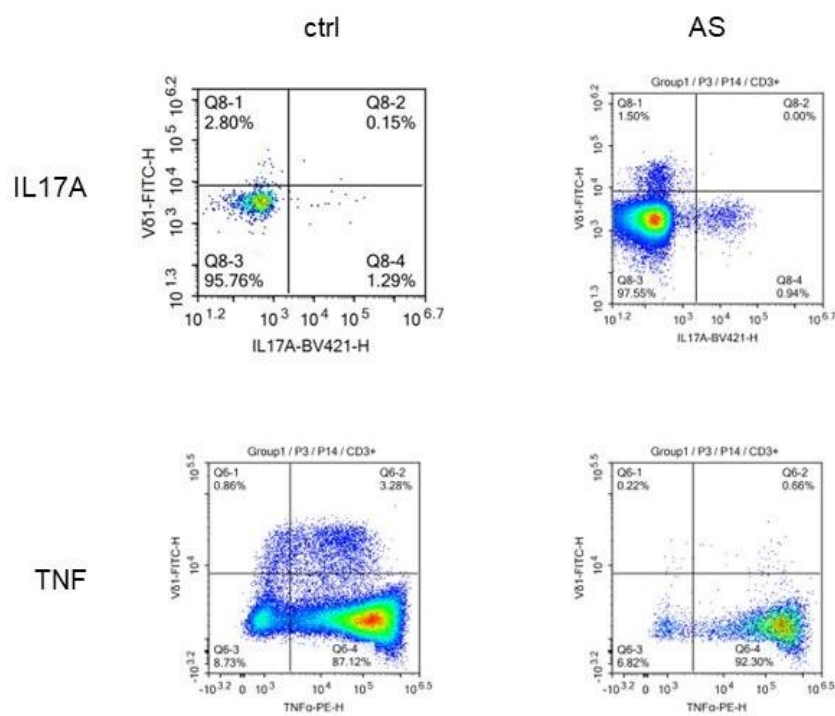
Subsets	AS (n = 17)	Healthy controls (n = 10)	P value
CD27+ $\gamma\delta$ T cells/V $\delta$ 1 T cells	0.567 $\pm$ 0.258	0.5310 $\pm$ 0.1848	0.703
CD27- $\gamma\delta$ T cells/V $\delta$ 1 T cells	0.2999 $\pm$ 0.1814	0.4329 $\pm$ 0.1900	0.091
CD27+ $\gamma\delta$ T cells/V $\delta$ 2 T	0.5173 $\pm$ 0.2781	0.7454 $\pm$ 0.1933	0.034
CD27- $\gamma\delta$ T cells/V $\delta$ 2 T cells	0.4767 $\pm$ 0.2750	0.2516 $\pm$ 0.1895	0.034
CD69-CD25+V $\delta$ 1 T cells/Parent	0.0321 (0.0185 – 0.044)	0.0157 (0.0091 – 0.0419)	0.209
CD69-CD25-V $\delta$ 1+ % Parent	0.7252 (0.5204 – 0.8507)	0.7104 (0.4844 – 0.7955)	0.912
CD69-CD25+V $\delta$ 2+ % Parent	0.0073 (0.0018 – 0.0353)	0.0071 (0.0029 – 0.0434)	0.407
CD69-CD25-V $\delta$ 2+ % Parent	0.6502 (0.2 – 0.9175)	0.8653 (0.6154 – 0.8868)	0.471
CD86-CD80+V $\delta$ 1+ % Parent	0.0318 (0.0011 – 0.0653)	0.0434 (0.0313 – 0.1289)	0.115
CD86-CD80+V $\delta$ 2+ % Parent	0.0003 (0 – 0.0018)	0 (0 – 0.0009)	0.856
CD86-CD80-V $\delta$ 1+ % Parent	0.9374 (0.8253 – 0.9589)	0.8599 (0.7921 – 0.9153)	0.155
CD86-CD80-V $\delta$ 2+ % Parent	0.9893 $\pm$ 0.0123	0.9841 $\pm$ 0.0084	0.369
CD69+CD25-V $\delta$ 1+ % Parent	0.2606 $\pm$ 0.1461	0.2785 $\pm$ 0.1617	0.727
CD69+CD25-V $\delta$ 2+ % Parent	0.275 (0.0756 – 0.72)	0.1175 (0.0959 – 0.3537)	0.802
CD69+CD25+V $\delta$ 1+ % Parent	0.0202 (0.007 – 0.0463)	0.0081 (0.002 – 0.0523)	0.292
CD69+CD25+V $\delta$ 2+ % Parent	0.0071 (0 – 0.0489)	0.0017 (0 – 0.0155)	0.283
CD86+CD80-V $\delta$ 1+ % Parent	0.0203 (0.0077 – 0.0924)	0.0454 (0.0242 – 0.1089)	0.156
CD86+CD80-V $\delta$ 2+ % Parent	0.0068 $\pm$ 0.0103	0.0155 $\pm$ 0.0087	0.092
CD86+CD80+V $\delta$ 1+ % Parent	0.0004 (0 – 0.014)	0.0133 (0.0079 – 0.03)	0.02
CD86+CD80+V $\delta$ 2+ % Parent	0 (0 – 0.001)	0 (0 – 0)	0.064

Note: AS, ankylosing spondylitis.

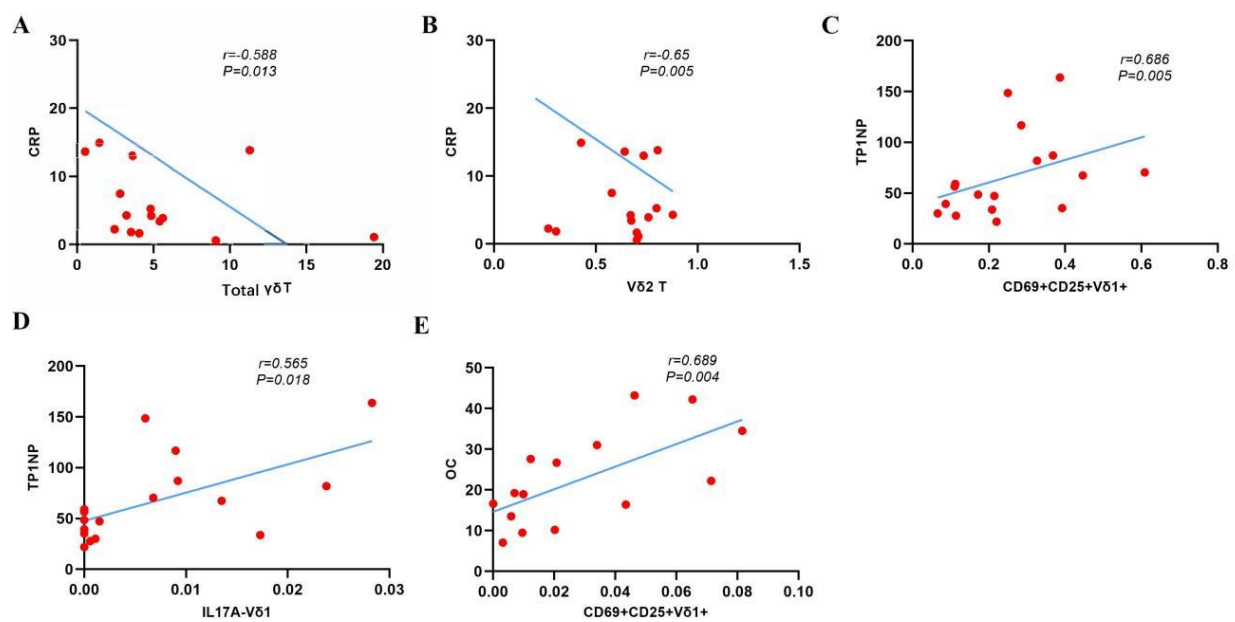
**Table 4.** Comparison of cytokine secretion of  $\gamma\delta$  T cells between AS patients and healthy controls.

Subsets	AS (n = 17)	Healthy controls (n = 10)	P value
IFN $\gamma$ -secreting V $\delta$ 1 cells/Parent	0.4941 $\pm$ 0.2634	0.4808 $\pm$ 0.1851	0.889
IFN $\gamma$ -secreting V $\delta$ 2 cells/Parent	0.5565 (0.3702 – 0.8757)	0.9242 (0.7034 – 0.9707)	0.056
IL17A-secreting V $\delta$ 1 cells/Parent	0.0015 (0 – 0.0114)	0.0105 (0.0042 – 0.0322)	0.04
IL17A- secreting V $\delta$ 2 cells/Parent	0 (0 – 0.0019)	0.0003 (0 – 0.0029)	0.44
TNF $\alpha$ - secreting V $\delta$ 1 cells/Parent	0.3150 $\pm$ 0.1490	0.4393 $\pm$ 0.1180	0.034
TNF $\alpha$ - secreting V $\delta$ 2 cells/Parent	0.3082 $\pm$ 0.1932	0.3751 $\pm$ 0.0875	0.231

Note: AS, ankylosing spondylitis.



**Fig. 3.** Flow cytometry analysis of the fractions of IL17A-secreting and TNF $\alpha$ -secreting V $\delta$ 1 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 V $\delta$ 1 and V $\delta$ 2 cells based on surface (CD3/V $\delta$ 2/V $\delta$ 1/CD25/CD69/CD27/CD80/CD86) or intracellular (CD3/v $\delta$ 2/v $\delta$ 1/CD27) marker expression. Cytokine secretion was measured using antibodies against TNF- $\alpha$ /IFN- $\gamma$ /IL-17A.



**Fig. 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 5.** Correlation of  $\gamma\delta$  T cell subsets with inflammation and bone turnover markers in AS patients.

Subsets	CRP		ESR		TP1NP		$\beta$ -CTx		OC		25(OH)VD3		PTH	
	r	P	r	P	r	P	r	P	r	P	r	P	r	P
$\gamma\delta$ 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
V $\delta$ 1 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
V $\delta$ 1 T cells/ $\gamma\delta$ 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
V $\delta$ 2 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
V $\delta$ 2 T cells/ $\gamma\delta$ 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+ $\gamma\delta$ T cells/V $\delta$ 1 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- $\gamma\delta$ T cells/V $\delta$ 1 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+ $\gamma\delta$ T cells/V $\delta$ 2 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- $\gamma\delta$ T cells/V $\delta$ 2 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 $\delta$ 1+	-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 $\delta$ 2+	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 $\delta$ 1+	-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
CD69-CD25-V $\delta$ 2+	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 $\delta$ 1+	-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 $\delta$ 2+	-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 $\delta$ 1+	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 $\delta$ 2+	-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 $\delta$ 1+	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 $\delta$ 2+	-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



Subsets	CRP		ESR		TP1NP		$\beta$ -CTx		OC		25(OH)VD3		PTH	
	r	P	r	P	r	P	r	P	r	P	r	P	r	P
CD69+CD25+V $\delta$ 1+	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 $\delta$ 2+	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 $\delta$ 1+	-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 $\delta$ 2+	-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 $\delta$ 1+	-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 $\delta$ 2+	-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
$\gamma\delta$ 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
V $\delta$ 1 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
V $\delta$ 1 T cells/ $\gamma\delta$ 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
V $\delta$ 2 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
V $\delta$ 2 T cells/ $\gamma\delta$ 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
IFN $\gamma$ -V $\delta$ 1	-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
IFN $\gamma$ -V $\delta$ 2	-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-V $\delta$ 1	-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-V $\delta$ 2	-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
TNF $\alpha$ -V $\delta$ 1	-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
TNF $\alpha$ -V $\delta$ 2	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;  $\beta$ -CTx,  $\beta$ -isomerized C-terminal telopeptides; TP1NP, procollagen type 1 amino-terminal propeptide; OC, osteocalcin; 25(OH)VD3, 25-hydroxyvitamin D3; PTH, parathyroid hormone.

## Discussion

In our study, peripheral V $\delta$ 2 T cells within the  $\gamma\delta$  T or CD3<sup>+</sup> T cell subpopulation in patients with AS were significantly decreased, whereas peripheral V $\delta$ 1 T cells within the  $\gamma\delta$  T cell subpopulation were significantly increased. These findings indicate a decreased V $\delta$ 2/V $\delta$ 1 ratio in the peripheral blood of patients with AS. Similarly, Tham *et al.* found a lower ratio of V $\delta$ 2/V $\delta$ 1 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 V $\delta$ 1 and V $\delta$ 2 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 V $\delta$ 1 cells, and only V $\delta$ 2 cells consistently expressed high levels of transcripts associated with the IL-23/IL-17 pathway [29]. In mice, IL-17A-producing  $\gamma\delta$  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 V $\delta$ 2 cells suggests that V $\delta$ 2 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  $\gamma\delta$  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<sup>+</sup> and CD8<sup>+</sup> T cells, and primed B cells, and  $\gamma\delta$  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<sup>+</sup>CD80<sup>+</sup> V $\delta$ 1 T cells, there were no significant differences in CD69, CD25, CD80, or CD86 expression in peripheral  $\gamma\delta$  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<sup>-</sup>  $\gamma\delta$  T cells [31]. Our data demonstrated that the proportion of CD27<sup>-</sup> cells in the V $\delta$ 2 subset was significantly higher in patients with AS than in healthy controls, suggesting that CD27<sup>-</sup> V $\delta$ 2 cells may be an important source of IL-17 in patients with AS. CD69 and CD25 are activation markers for T cells [27].  $\gamma\delta$  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- $\alpha$ . 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<sup>+</sup>V $\delta$ 2 cells and disease activity has a slight tendency toward statistical significance [28]. In addition, Zhao *et al.* demonstrated that the number of circulating CD4<sup>+</sup>CD25<sup>high</sup>CD127<sup>low</sup>/- 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  $\gamma\delta$  T cell subpopulations may indicate the severity of AS and our study made several attempts. Previous reports indicated that inflammatory cytokines IL-17A, TNF- $\alpha$ , and IFN $\gamma$  are involved in the pathophysiology of AS [26];  $\gamma\delta$  T cells express IL-1, IL-6, IL-18, IL-23, and TGF $\beta$ 1 receptors to stimulate IL-17 production;  $\gamma\delta$  T cells also release additional proinflammatory cytokines, such as TNF $\alpha$  and IFN $\gamma$  [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];  $\gamma\delta$  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 V $\delta$ 1 cells secreting IL-17A or TNF $\alpha$  in patients with AS relative to healthy controls. Other cytokine-producing T cells exhibited no distinguishing characteristics. Th17, IL-17-producing CD8<sup>+</sup> T cells, type 3 innate lymphoid cells, and  $\gamma\delta$  T cells may produce IL-17 as a result of IL-23 stimulation [41]. In our study, the decrease in V $\delta$ 1 cells that secrete IL-17A or TNF $\alpha$  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  $\gamma\delta$  T cells/PBMCs and CRP, V $\delta$ 2 cells/ $\gamma\delta$  T cells and CRP, V $\delta$ 2 cells/CD3<sup>+</sup> T cells and CRP, and V $\delta$ 1 cells/CD3<sup>+</sup> T cells and ESR. Similarly, Mo *et al.* found that peripheral V $\delta$ 2 T cells but not V $\delta$ 1 T cells, were significantly lower in patients with RA and negatively correlated with disease activity [44]. The V $\delta$ 2 T cells may accumulate in inflamed tissue because they produce high levels of proinflammatory cytokines, including IL-17, TNF- $\alpha$ , and IFN- $\gamma$ . V $\delta$ 1 cells/ $\gamma\delta$  T cells and CRP,  $\gamma\delta$  T cells/PBMCs and  $\beta$ -CTX, CD69<sup>+</sup>CD25<sup>+</sup> or IL17A-secreting V $\delta$ 1 cells and TP1NP, as well as CD69<sup>+</sup>CD25<sup>+</sup> V $\delta$ 1 and V $\delta$ 2 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  $\gamma\delta$  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  $\gamma\delta$  T cells in patients with AS, but the changes in the  $\gamma\delta$  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  $\gamma\delta$  T subpopulations in patients with active versus in remission AS. Second, due to the scarcity of enthesal  $\gamma\delta$  T cells, there is insufficient data to study the composition of  $\gamma\delta$  T cells at the site of inflammation and to characterize the functions of  $\gamma\delta$  T cell subsets in the development of AS. It is necessary to investigate the distribution of  $\gamma\delta$  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 outlier observations.

## Conclusions

In this study, we demonstrated that patients with AS had significantly fewer V $\delta$ 2 cells than healthy controls.

## References

1. Dean LE, Jones GT, MacDonald AG, Downham C, Sturrock RD, Macfarlane GJ. Global prevalence of ankylosing spondylitis. *Rheumatology (Oxford)*. 2014;53:650-657. <https://doi.org/10.1093/rheumatology/ket387>

In particular, CD27<sup>+</sup> V $\delta$ 2 T cells, CD86<sup>+</sup>CD80<sup>+</sup> V $\delta$ 1 T cells, and IL17A<sup>-</sup> and TNF $\alpha$ -secreting V $\delta$ 1 T cells were reduced in patients with AS. These factors may contribute to the pathogenesis of AS. Furthermore, the fractions of  $\gamma\delta$  T cells in PBMCs, V $\delta$ 2 cells in  $\gamma\delta$  T cells, as well as V $\delta$ 1 or V $\delta$ 2 cells in CD3<sup>+</sup> T cells negatively correlated with CRP, suggesting that the imbalance in  $\gamma\delta$  T cell subpopulations may reflect the severity of the disease. We also identified a CD69<sup>+</sup>CD25<sup>+</sup> subpopulation and observed significant positive correlations between the CD69<sup>+</sup>CD25<sup>+</sup> subpopulation and bone turnover markers, suggesting that CD69<sup>+</sup>CD25<sup>+</sup>  $\gamma\delta$  T cells regulate bone turnover and contribute to the pathogenesis of AS.

## Conflict of Interest

There is no conflict of interest.

## Acknowledgements

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.

The work was funded by Beijing Natural Science Foundation(L202011); Beijing JST Research Funding (ZR-201915) and Beijing JST Research Funding (ZR-202223).

## Abbreviations

$\gamma\delta$  T cell, gamma delta T cell; AS, ankylosing spondylitis; SpA, spondyloarthritis; HLA, Human leukocyte antigen; TCR, T-cell receptor; IFN- $\gamma$ , interferon- $\gamma$ ; TNF- $\alpha$ , tumor necrosis factor  $\alpha$ ; 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;  $\beta$ -CTX,  $\beta$ -isomerized C-terminal telopeptides; TP1NP, procollagen type 1 amino-terminal propeptide; OC, osteocalcin; 25(OH)VD3, 25-hydroxyvitamin D3; PTH, parathyroid hormone; IL, interleukin;

2. Taurog JD, Chhabra A, Colbert RA. Ankylosing spondylitis and axial spondyloarthritis. *N Engl J Med*. 2016;374:2563-2574. <https://doi.org/10.1056/NEJMra1406182>
3. Australo-Anglo-American Spondyloarthritis Consortium, Reveille JD, Sims AM, Danoy P, Evans DM, Leo P, Pointon JJ, Zhou X, Bradbury LA, Appleton LH, Davis JC Jr, Diekman L, Doan T, Schulz Duan R, Duncan EL, Farrar C, Hadler J, Harvey D et al. Genome-wide association study of ankylosing spondylitis identifies non-MHC susceptibility loci. *Nat Genet*. 2010;42:123-127. <https://doi.org/10.1038/ng.513>
4. Meliconi R., Pitzalis C., Kingsley G.H., and Panayi G.S. Gamma/delta T cells and their subpopulations in blood and synovial fluid from rheumatoid arthritis and spondyloarthritis. *Clin Immunol Immunopathol*. 1991;59:165-172. [https://doi.org/10.1016/0090-1229\(91\)90090-W](https://doi.org/10.1016/0090-1229(91)90090-W)
5. Kenna TJ, Davidson SI, Duan R, Bradbury LA, McFarlane J, Smith M, Weedon H, Street S, Thomas R, Thomas GP, Brown MA. Enrichment of circulating interleukin-17-secreting interleukin-23 receptor-positive gamma/delta T cells in patients with active ankylosing spondylitis. *Arthritis Rheum*. 2012;64(7):1420-1429. <https://doi.org/10.1002/art.33507>
6. Adams EJ, Gu S, Luoma AM. Human gamma delta T cells: Evolution and ligand recognition. *Cell Immunol*. 2015;296:31-40. <https://doi.org/10.1016/j.cellimm.2015.04.008>
7. Nielsen MM, Witherden DA, Havran WL. gammadelta T cells in homeostasis and host defence of epithelial barrier tissues. *Nat Rev Immunol*. 2017;17:733-745. <https://doi.org/10.1038/nri.2017.101>
8. Gu S, Nawrocka W, Adams EJ. Sensing of Pyrophosphate Metabolites by Vgamma9Vdelta2 T Cells. *Front Immunol*. 2014;5:688. <https://doi.org/10.3389/fimmu.2014.00688>
9. Vantourout P, Hayday A. Six-of-the-best: unique contributions of gammadelta T cells to immunology. *Nat Rev Immunol*. 2013;13:88-100. <https://doi.org/10.1038/nri3384>
10. Sutton CE, Lalor SJ, Sweeney CM, Brereton CF, Lavelle EC, Mills KHG. Interleukin-1 and IL-23 induce innate IL-17 production from gammadelta T cells, amplifying Th17 responses and autoimmunity. *Immunity*. 2009;31:331-341. <https://doi.org/10.1016/j.immuni.2009.08.001>
11. Lawand M, Dechanet-Merville J, Dieu-Nosjean MC. Key features of gamma-delta T-cell subsets in human diseases and their immunotherapeutic implications. *Front Immunol*. 2017;8:761. <https://doi.org/10.3389/fimmu.2017.00761>
12. Ono T, Okamoto K, Nakashima T, Nitta T, Hori S, Iwakura Y, Takayanagi H. IL-17-producing gamma delta T cells enhance bone regeneration. *Nat Commun*. 2016;7:10928. <https://doi.org/10.1038/ncomms10928>
13. Baeten D, Sieper J, Braun J, Baraliakos X, Dougados M, Emery P, Deodhar A, Porter B, Martin R, Andersson M, Mpfu S, Richards HB, Group MS, Group MS. Secukinumab, an Interleukin-17A Inhibitor, in Ankylosing Spondylitis. *N Engl J Med*. 2015;373(26):2534-2548. <https://doi.org/10.1056/NEJMoa1505066>
14. Dubash S, Bridgwood C, McGonagle D, Marzo-Ortega H. The advent of IL-17A blockade in ankylosing spondylitis: secukinumab, ixekizumab and beyond. *Expert Rev Clin Immunol*. 2019;15:123-134. <https://doi.org/10.1080/1744666X.2019.1561281>
15. Jo S, Kang S, Han J, Choi SH, Park YS, Sung IH, Kim TH. Accelerated osteogenic differentiation of human bone-derived cells in ankylosing spondylitis. *J Bone Miner Metab*. 2018;36:307-313. <https://doi.org/10.1007/s00774-017-0846-3>
16. Pang DJ, Neves JF, Sumaria N, Pennington DJ. Understanding the complexity of gammadelta T-cell subsets in mouse and human. *Immunology*. 2012;136:283-290. <https://doi.org/10.1111/j.1365-2567.2012.03582.x>
17. Raphael I, Nalawade S, Eagar TN, Forsthuber TG. T cell subsets and their signature cytokines in autoimmune and inflammatory diseases. *Cytokine*. 2015;74:5-17. <https://doi.org/10.1016/j.cyto.2014.09.011>
18. van der Linden S, Valkenburg HA, Cats A. Evaluation of diagnostic criteria for ankylosing spondylitis. A proposal for modification of the New York criteria. *Arthritis Rheum*. 1984;27:361-368. <https://doi.org/10.1002/art.1780270401>
19. Puleo A, Carroll C, Maecker HT, Gupta R. Isolation of Peripheral Blood Mononuclear Cells Using Vacutainer(R) Cellular Preparation Tubes (CPT(TM)). *Bio Protoc*. 2017;7:e2103. <https://doi.org/10.21769/BioProtoc.2103>
20. Wu Z, Zheng Y, Sheng J, Han Y, Yang Y, Pan H, Yao J. CD3(+)CD4(-)CD8(-) (Double-Negative) T Cells in Inflammation, Immune Disorders and Cancer. *Front Immunol*. 2022;13:816005. <https://doi.org/10.3389/fimmu.2022.816005>

21. Fuse S, Obar JJ, Bellfy S, Leung EK, Zhang W, Usherwood EJ. CD80 and CD86 control antiviral CD8+ T-cell function and immune surveillance of murine gammaherpesvirus 68. *J Virol.* 2006;80:9159-9170. <https://doi.org/10.1128/JVI.00422-06>
22. Ferenczi K, Burack L, Pope M, Krueger JG, Austin LM. CD69, HLA-DR and the IL-2R identify persistently activated T cells in psoriasis vulgaris lesional skin: blood and skin comparisons by flow cytometry. *J Autoimmun.* 2000;14(1):63-78. <https://doi.org/10.1006/jaut.1999.0343>
23. Antony PA, Restifo NP. CD4+CD25+ T regulatory cells, immunotherapy of cancer, and interleukin-2. *J Immunother.* 2005;28(2):120-128. <https://doi.org/10.1097/01.cji.0000155049.26787.45>
24. Ewing MM, Karper JC, Abdul S, de Jong RC, Peters HA, de Vries MR, Redeker A, Kuiper J, Toes RE, Arens R, Jukema JW, Quax PH. T-cell co-stimulation by CD28-CD80/86 and its negative regulator CTLA-4 strongly influence accelerated atherosclerosis development *Int J Cardiol* 2013;168:1965-1974. <https://doi.org/10.1016/j.ijcard.2012.12.085>
25. Reveille JD. Biomarkers for diagnosis monitoring of progression and treatment responses in ankylosing spondylitis and axial spondyloarthritis *Clin Rheumatol* 2015;34(10):1009-1018. <https://doi.org/10.1007/s10067-015-2949-3>
26. Chisalau BA, Cringus LI, Vreju FA, Parvanescu CD, Firulescu SC, Dinescu SC, Ciobanu DA, Tica AA, Sandu RE, Silosi I, Boldeanu MV, Poenariu IS, Ungureanu AM, Boldeanu L, Barbulescu AL. New insights into IL-17/IL-23 signaling in ankylosing spondylitis (Review) *Exp Ther Med* 2020;20(3):3493-3497. <https://doi.org/10.3892/etm.2020.8981>
27. Hosono M, de Boer OJ, van der Wal AC, van der Loos CM, Teeling P, Piek JJ, Ueda M, Becker AE. Increased expression of T cell activation markers (CD25 CD26 CD40L and CD69) in atherectomy specimens of patients with unstable angina and acute myocardial infarction *Atherosclerosis* 2003;168:73-80. [https://doi.org/10.1016/S0021-9150\(03\)00024-8](https://doi.org/10.1016/S0021-9150(03)00024-8)
28. Tham M, Schlor GR, Yerly D, Mueller C, Surbek D, Villiger PM, Forger F. Reduced pro-inflammatory profile of gammadeltaT cells in pregnant patients with rheumatoid arthritis *Arthritis Res Ther* 2016;18(1):26. <https://doi.org/10.1186/s13075-016-0925-1>
29. Cuthbert RJ, Watad A, Fragkakis EM, Dunsmuir R, Loughenbury P, Khan A, Millner PA, Davison A, Marzo-Ortega H, Newton D, Bridgewood C, McGonagle DG. Evidence that tissue resident human enthesitis gammadeltaT-cells can produce IL-17A independently of IL-23R transcript expression *Ann Rheum Dis* 2019;78(12):1559-1565. <https://doi.org/10.1136/annrheumdis-2019-215210>
30. Reinhardt A, Yevsa T, Worbs T, Lienenklaus S, Sandrock I, Oberdorfer L, Korn T, Weiss S, Forster R, Prinz I. Interleukin-23-Dependent gamma/delta T Cells Produce Interleukin-17 and Accumulate in the Enthesis Aortic Valve and Ciliary Body in Mice Arthritis. *Rheumatol* 2016;68(10):2476-2486. <https://doi.org/10.1002/art.39732>
31. Ribot JC, deBarros A, Pang DJ, Neves JF, Peperzak V, Roberts SJ, Girardi M, Borst J, Hayday AC, Pennington DJ, Silva-Santos B. CD27 is a thymic determinant of the balance between interferon-gamma- and interleukin 17-producing gammadelta T cell subsets. *Nat Immunol.* 2009;10(4):427-436. <https://doi.org/10.1038/ni.1717>
32. Brandes M, Willmann K, Moser B. Professional antigen-presentation function by human gammadelta T Cells. *Science.* 2005;309(5732):264-268. <https://doi.org/10.1126/science.1110267>
33. Hayashi E, Chiba A, Tada K, Haga K, Kitagaichi M, Nakajima S, Kusaoi M, Sekiya F, Ogasawara M, Yamaji K, Tamura N, Takasaki Y, Miyake S. Involvement of mucosal-associated invariant T cells in ankylosing spondylitis *J Rheumatol* 2016;43(10):1695-1703. <https://doi.org/10.3899/jrheum.151133>
34. Zhao SS, Hu JW, Wang J, Lou XJ, Zhou LL. Inverse correlation between CD4+ CD25high CD127low/- regulatory T-cells and serum immunoglobulin A in patients with new-onset ankylosing spondylitis *J Int Med Res* 2011;39(6):1968-1974. <https://doi.org/10.1177/147323001103900543>
35. DuWL, Ba YN, Lv TT, Zheng ZH, Li XY, Ding J, Xiao GZ, Li Y, Xie RH, Zhu P, Yang XC, Wu ZB. The expression and significance of CD28 Ctl-4 CD80 and CD86 in ankylosing spondylitis were also stimulated. *Biomed Res* 2018;29. <https://doi.org/10.4066/biomedicalresearch.29-17-2704>
36. Odobasic D, Leech MT, Xue JR, Holdsworth SR. Distinct in vivo roles of CD80 and CD86 in the effector T-cell responses inducing antigen-induced arthritis *Immunology* 2008;124(4):503-513. <https://doi.org/10.1111/j.1365-2567.2007.02802.x>

- 
37. Wang Y, Rothstein TL. Induction of Th17 cell differentiation by B-1 cells *Front Immunol* 2012;3:281. <https://doi.org/10.3389/fimmu.2012.00281>
  38. Rosine N, Miceli-Richard C. Innate Cells: The Alternative Source of IL-17 in Axial and Peripheral Spondyloarthritis? *Front Immunol*. 2020;11:553742. <https://doi.org/10.3389/fimmu.2020.553742>
  39. Shen H, Goodall JC, Hill Gaston JS. Frequency and phenotype of peripheral blood Th17 cells in ankylosing spondylitis and rheumatoid arthritis. *Arthritis Rheum*. 2009;60:1647-1656. <https://doi.org/10.1002/art.24568>
  40. Zhang L, Li YG, Li YH, Qi L, Liu XG, Yuan CZ, Hu NW, Ma DX, Li ZF, Yang Q, Li W, Li JM. Increased frequencies of Th22 cells as well as Th17 cells in the peripheral blood of patients with ankylosing spondylitis and rheumatoid arthritis. *PLoS One*. 2012;7(11):e31000. <https://doi.org/10.1371/journal.pone.0031000>
  41. Tsukazaki H, Kaito T. The Role of the IL-23/IL-17 Pathway in the Pathogenesis of Spondyloarthritis. *Int J Mol Sci*. 2020;21(17):6401. <https://doi.org/10.3390/ijms21176401>
  42. Bray C, Bell LN, Liang H, Haykal R, Kaiksow F, Mazza JJ, Yale SH. Erythrocyte Sedimentation Rate and C-reactive Protein Measurements and Their Relevance in Clinical Medicine. *WMJ*. 2016;115(6):317-321.
  43. Hu T, Yang Q, Xu J, Zhang Z, He N, Du Y. Role of beta-isomerized C-terminal telopeptides (beta-CTX) and total procollagen type 1 amino-terminal propeptide (tP1NP) as osteosarcoma biomarkers. *Int J Clin Exp Med*. 2015;8(1):890-896.
  44. Mo WX, Yin SS, Chen H, Zhou C, Zhou JX, Zhao LD, Fei YY, Yang HX, Guo JB, Mao YJ, Huang LF, Zheng WJ, Zhang W, Zhang JM, He W, Zhang X. Chemotaxis of Vdelta2 T cells to the joints contributes to the pathogenesis of rheumatoid arthritis *Ann Rheum Dis* 2017;76(12):2075-2084. <https://doi.org/10.1136/annrheumdis-2016-211069>
  45. Haroon N. Ankylosis in ankylosing spondylitis: current concepts. *Clin Rheumatol* 2015;34(10):1003-1007. <https://doi.org/10.1007/s10067-015-2956-4>
-