Concise report

Potential role of Th17 cells in the pathogenesis of adult-onset Still’s disease

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Abstract

Objective. To investigate the potential role of Th type 17 (Th17) cells and Th17-related cytokines in the pathogenesis of adult-onset Still’s disease (AOSD).

Methods. The frequencies of circulating Th17 cells in 24 patients with active untreated AOSD, 16 patients with active SLE and 12 healthy volunteers were determined using intracellular cytokine staining and flow cytometry. Serum levels of Th17-related cytokines, including IL-1β, IL-6, IL-17, IL-18, IL-21 and IL-23 were measured by ELISA.

Results. Significantly higher median frequencies of circulating Th17 cells were found in active untreated AOSD patients (1.01%) and active SLE patients (1.26%) than in healthy volunteers (0.12%, both \( P < 0.001 \)). The frequencies of circulating Th17 cells were positively correlated with activity score \((r = 0.527, P < 0.01)\) and serum ferritin levels \((r = 0.724, P < 0.001)\) in AOSD patients, and correlated with SLEDAI \((r = 0.663, P < 0.01)\) in SLE patients. Additionally, the frequencies of circulating Th17 cells were positively and significantly correlated with serum levels of IL-1β, IL-6, IL-17, IL-18, IL-21 and IL-23 in both AOSD and SLE patients. The frequencies of circulating Th17 cells and serum IL-17 levels significantly decreased after effective therapy in AOSD patients (both \( P < 0.001 \)).

Conclusion. Elevated frequencies of circulating Th17 cells and a positive correlation with disease activity in our AOSD patients suggest that Th17 cells contribute to the pathogenesis of this disease. Dysregulation of Th17 cells may be a common pathogenic mechanism that underlies the development of both AOSD and SLE.

Key words: T helper type 17 cells, T helper type 17-related cytokines, Pathogenesis, Adult-onset Still’s disease, Systemic lupus erythematosus.

Introduction

T helper (Th) type 17 (Th17) cells, a novel distinct subset of Th cell, can secrete IL-17 in humans [1–4]. IL-17 (also known as IL-17A) is a pleiotropic cytokine that participates in tissue inflammation by inducing the expression of pro-inflammatory cytokines, chemokines and MMPs, which mediate inflammatory cell infiltration and tissue destruction [1, 5–7]. Elevated levels of IL-17 have been detected in sera and enhanced expression of IL-17 has been observed in target tissues of patients with various autoimmune diseases, including SLE and RA [8–12]. Moreover, IL-17 has become the new therapeutic target for animal models with collagen-induced arthritis as well as autoimmune uveoretinitis, and other autoimmune diseases [13–15]. Therefore, Th17 cells play a critical role in autoimmune diseases.

Adult-onset Still’s disease (AOSD) is an inflammatory autoimmune disorder, characterized by high spiking fever, evanescent rash, arthritis, hepatosplenomegaly, variable multisystemic involvement and laboratory abnormalities that include neutrophilic leucocytosis and hyperferritinaemia [16, 17]. Our previous studies and other reports have shown that pro-inflammatory cytokines

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including IL-1β, IL-6 and IL-18 play a vital role in the pathogenesis of AOSD [18–21]. Recent studies show that IL-1β and IL-6 induce the differentiation of Th17 cells from naïve T cells [22–23]. IL-21 plays a key amplification role in the generation of Th17 cells [24–25] and IL-23 has ability to maintain Th17 responses in human [26, 27]. IL-18 synergizes with IL-23 in the production of IL-17-producing CD4 T cells [28]. Moreover, Wong et al. [10] indicated that IL-18 could significantly induce the ex vivo release of IL-17 and IL-23 in SLE patients [10]. Therefore, we hypothesize that Th17 cells may play a potential role in the pathogenesis of AOSD. However, there are no data concerning the associations of Th17 cells and their cytokine profiles with disease activity in AOSD patients.

In this study, Th17 cells were quantified in peripheral blood (PB) from AOSD patients and SLE patients using intracellular cytokine staining and flow cytometry analysis. We enrolled SLE patients as disease control because of some similarities in systemic manifestations between SLE and AOSD and because a number of previous studies have documented Th17-related cytokines in SLE [9, 10, 29, 30]. The correlations between the frequencies of circulating Th17 cells as well as serum IL-17 levels and disease activity were investigated in both AOSD patients and SLE patients. The changes in the frequencies of circulating Th17 cells and the levels of serum IL-17 during longitudinal follow-up of AOSD patients were also studied.

Methods

Patients

Twenty-four patients with active untreated AOSD [18 females and 6 males, mean (± s.d.) 37.3 (14.0) years] fulfilling the Yamaguchi criteria [31] were enrolled. Patients with infections, malignancies or other rheumatic diseases were excluded. The DASs (range 0–12) for each AOSD patient were assessed according to the criteria described by Pouchot et al. [32]. After initial investigation for Th17 cells and Th17-related cytokines, all AOSD patients received CSs and NSAIDs. The DMARDs used were MTX (18 patients), HCQ (17 patients), SSZ (8 patients) and AZA (4 patients). Sixteen age-matched patients [14 females and 2 males, mean age 35.0 (12.9 years)] fulfilling the 1997 revised criteria of the ACR for SLE [33] were included as disease controls for systemic inflammation. Disease activity of SLE was determined by calculating the SLEDAI [34]. All SLE patients who had poor drug compliance did not receive CS or immunosuppressive agents at least 1 month before enrolment in this study. Twelve age-matched healthy volunteers [10 females and 2 males, mean age 35.5 (13.9 years)], who had no rheumatic disease, were used as normal controls. Peripheral blood was collected using endotoxin-free heparinized vacuum tubes (KABI-ET; Chromogenix, Antwerp, Belgium) to avoid cytokine production during the interval between sampling and culture. The Ethics Committee of Clinical Research, Taichung Veterans General Hospital (IRB TCVGH No: C08113), approved the study and informed consent was obtained from each participant according to the Declaration of Helsinki.

Quantification of circulating Th17 cells using flow cytometry analysis

For detection of circulating Th17 cells, phycoerythrin (PE)-conjugated anti-IL-17 (eBioscience, San Diego, CA, USA) and phycoerythrin–cyanin 5 (PC5)-conjugated anti-CD4 (Beckman Coulter, Marseilles, France) were quantified using flow-cytometric analysis according to the manufacturer’s protocol and the described technique [35, 36]. Briefly, aliquots of 1000 µl of the sterile heparinized whole blood were stimulated with a combination of 25 ng/ml phorbol myristate acetate (PMA) and 1 µg/ml of ionomycin (Sigma, Deisenhofen, Germany) and cultured for 1 h at 37°C in a humidified 5% CO2 incubator. Whole blood was then treated with 10 µg/ml of Brefeldin A (BFA; Sigma) to inhibit intracellular protein transport. Activated cultures of blood samples were washed in wash buffer (PBS, 5% fetal bovine serum, 0.1% sodium azide; Merck, Darmstadt, Germany) and then stained with 20 µl of PC5-conjugated CD4-specific mAb (Beckman Coulter, Marseilles, France) for 15 min at room temperature (RT). Erythrocytes were lysed by adding 2 ml of FACS lysing solution (Becton Dickinson, Lincoln Park, NJ, USA). After 5 min incubation, samples were centrifuged and washed with 0.1% BSA–PBS, and subsequently fixed with 100 µl of Reagent 1 (Beckman Coulter, USA) for 10 min. After washing, the pellet was incubated with 100 µl of Reagent 2, saponin (Beckman Coulter, Marseilles, France) for 5 min at RT in the dark. The samples were washed twice with 0.1% BSA–PBS, and incubated with PE-conjugated IL-17-specific mAb (eBiosciences) for 30 min at RT in the dark. An isotype control IgG1-PE (eBiosciences) was used for the IL-17 staining at RT in the dark. After staining, the cells were washed and immediately analysed using flow cytometry (Beckman Coulter, Miami, FL, USA). Lymphocytes were gated on the basis of forward- and side-scatter properties and at least 10,000 CD4+ cells were analysed. Data were obtained using an Epics flow cytometer, and the results were analysed using Expo32 software (Beckman Coulter, Miami, FL, USA).

Determination of serum levels of Th17-related cytokines

Serum levels of IL-1β, IL-6, IL-17, IL-18, IL-21 and IL-23 were determined in AOSD patients, SLE patients and healthy controls using ELISA according to the manufacturer’s instructions (eBiosciences).

Statistical analysis

Results are presented as the mean (± s.d.) or median [inter-quartile range (IQR)]. The non-parametric Kruskal–Wallis test was used for between-group comparison of serum levels of Th17-related cytokines, and the frequencies of circulating Th17 cells. When this test showed significant differences, then the exact P-values were determined using the Mann–Whitney U-test. The correlation coefficient was obtained by the non-parametric
Spearman’s rank correlation test. For comparison of the frequencies of circulating Th17 cells and serum levels of IL-17 during follow-up for AOSD patients after effective therapy, the Wilcoxon signed-rank test was employed. \( P < 0.05 \) was considered to be statistically significant.

**Results**

Clinical characteristics of AOSD patients and SLE patients

As illustrated in Table 1, all 24 patients with active untreated AOSD had daily spiking fevers (≥38°C). Evanescent rash and sore throat were present in 20 (83.3%) patients. Arthritis, lymphadenopathy and hepatomegaly were noted in 16 (66.7%), 11 (45.8%) and 8 (33.3%) patients, respectively. All SLE patients had active disease [mean SLEDAI (s.d.), 16.3 (4.1) and range 9–24)] at the time of investigation and 10 (62.5%) patients had renal involvement. However, there were no significant differences in the age at onset, the proportion of female patients or the frequencies of extra-renal manifestations between AOSD patients and SLE patients.

The percentage of circulating Th17 cells in AOSD patients and SLE patients

Representative examples of flow cytometric dot-plots of intracellular IL-17 production in Th cells obtained from PB of one patient with active untreated AOSD (Fig. 1A), a patient with active SLE (Fig. 1B) and a healthy control (Fig. 1C) are shown. Significantly higher median frequencies of circulating Th17 cells were observed in patients with active untreated AOSD [median (IQR) 1.01 (0.45–2.26%)] and in patients with active SLE [median (IQR) 1.26 (0.80–1.88%)] than in healthy controls [median (IQR) 0.12 (0.05–0.18%); both \( P < 0.001 \)]. However, there was no significant difference in the percentage of circulating Th17 cells between AOSD patients and SLE patients.

Serum levels of Th17-related cytokines in AOSD patients and SLE patients

As shown in Fig. 2, serum levels of IL-17, IL-6, IL-18, IL-21 and IL-23 in both AOSD patients and SLE patients. The frequencies of circulating Th17 cells were significantly and positively correlated with serum levels of IL-23 in healthy controls. Serum levels of IL-17 from AOSD patients were also positively correlated with the levels of serum IL-17 in SLE patients. IL-18, IL-21 and IL-23 both at the active phase and at the remission phase. As illustrated in Table 2, the frequencies of circulating Th17 cells and serum levels of IL-17 significantly decreased (median 1.37 vs 0.77 pg/ml, respectively; both \( P < 0.001 \)), paralleling clinical remission and the decrease in serum levels of ferritin (515.0 vs 214.5 µg/l, \( P < 0.001 \)) in AOSD patients after effective therapy.

**Correlation between frequencies of circulating Th17 cells as well as serum IL-17 levels and DASs in AOSD patients and SLE patients**

The frequencies of circulating Th17 cells and the levels of serum IL-17 from AOSD patients were positively correlated with DASs \( (r = 0.527, P < 0.01 \) and \( r = 0.565, P < 0.005, \) respectively) and with serum ferritin levels \( (r = 0.724 \) and \( r = 0.829, \) respectively, both \( P < 0.001 \)). Similarly, the frequencies of circulating Th17 cells and the levels of serum IL-17 from SLE patients were positively correlated with SLEDAI \( (r = 0.663, P < 0.01 \) and \( r = 0.708, P < 0.005, \) respectively).

**Correlation between the frequencies of circulating Th17 cells and serum levels of Th17-related cytokines in AOSD patients and SLE patients**

As illustrated in Table 2, the frequencies of circulating Th17 cells and the levels of serum IL-17, IL-6, IL-18, IL-21 and IL-23 in both AOSD patients and SLE patients. The frequencies of circulating Th17 cells were positively correlated with serum levels of IL-23 in healthy controls. Serum levels of IL-17 from AOSD patients were also positively correlated with the levels of serum IL-17 \( (r = 0.533, P < 0.01) \), IL-18 \( (r = 0.669, P < 0.001) \), IL-21 \( (r = 0.707, P < 0.001) \) and IL-23 \( (r = 0.815, P < 0.001) \).

**Changes in the levels of circulating Th17 cells and serum IL-17 in AOSD patients after effective therapy**

Twenty AOSD patients were available for examination both at the active phase and at the remission phase. As illustrated in Fig. 3, the frequencies of circulating Th17 cells and the levels of serum IL-17 significantly decreased (median 1.37 vs 0.16%); 5.77 vs 2.77 pg/ml, respectively, both \( P < 0.001 \), paralleling clinical remission and the decrease in serum levels of ferritin (515.0 vs 214.5 µg/l, \( P < 0.001 \)) in AOSD patients after effective therapy.

**Table 1** Demographic data and clinical characteristics of patients with AOSD, patients with SLE and healthy controls

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>AOSD (n = 24)</th>
<th>SLE (n = 16)</th>
<th>HC (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at study entry, mean (s.d.), years</td>
<td>37.3 (14.0)</td>
<td>35.0 (12.9)</td>
<td>35.5 (13.9)</td>
</tr>
<tr>
<td>Proportion of females</td>
<td>18 (75.0)</td>
<td>14 (87.5)</td>
<td>10 (83.3)</td>
</tr>
<tr>
<td>Fever, ≥38°C</td>
<td>24 (100)</td>
<td>13 (81.3)</td>
<td>NA</td>
</tr>
<tr>
<td>Rash</td>
<td>20 (83.3)</td>
<td>12 (75.0)</td>
<td>NA</td>
</tr>
<tr>
<td>Arthritis</td>
<td>16 (66.7)</td>
<td>10 (62.5)</td>
<td>NA</td>
</tr>
<tr>
<td>Lymphadenopathy</td>
<td>11 (45.8)</td>
<td>6 (37.5)</td>
<td>NA</td>
</tr>
<tr>
<td>Hepatosplenomegaly</td>
<td>8 (33.3)</td>
<td>3 (18.8)</td>
<td>NA</td>
</tr>
<tr>
<td>Nephritis</td>
<td>0 (0.0)</td>
<td>10 (62.5)</td>
<td>NA</td>
</tr>
</tbody>
</table>

Data are presented as n (%) unless otherwise mentioned. Nephritis is defined by persistent proteinuria (≥0.5 g/24 h), the presence of cellular casts or pathological examination of renal biopsy specimens showing lupus nephritis. NA: not applicable; HC: healthy control.
Discussion

This study is the first attempt to investigate the frequency of circulating Th17 cells in active untreated AOSD patients. In order to obtain a better reflection of in vivo cytokine patterns than is achievable with mononuclear cells from PB, whole blood was stimulated with mitogens and the double-stained lymphocytes with IL-17 and CD4 were analysed using flow cytometry. To avoid the effects of CSs and other immunosuppressive agents on the results of circulating Th17 cells, new-onset untreated patients with AOSD were enrolled. Our data showed that significantly higher frequencies of circulating Th17 cells were found in active AOSD patients than in healthy controls. Moreover, a parallel decrease in the frequency of circulating Th17 cells with disease remission was found in our AOSD patients. Our results strongly imply a potential role for Th17 cells in the pathogenesis of AOSD.

Similar to AOSD patients, SLE patients had significantly elevated frequencies of circulating Th17 cells and these elevated frequencies were correlated with disease activity. Our results were consistent with the findings of recent studies showing elevated frequencies of Th17 cells using the enzyme-linked immunosorbent spot (ELISPOT) assay [10] and using flow cytometry assay in SLE patients [29], and augmented mRNA expression of retinoic acid receptor-related orphan receptor (ROR)-γt, which is the specific transcription factor for Th17 cells [30]. These observations suggested that elevated frequencies of circulating Th17 cells may be a common characteristic of systemic inflammatory diseases including AOSD and SLE.

Th17 cells have a specific role in immune function through the production of effector cytokines. We found a positive correlation between frequencies of circulating Th17 cells and serum IL-17 levels in both AOSD and SLE patients. Although a recent study showed that proportionally, mast cells and not Th17 cells appear to be a
major source of IL-17 in rheumatoid synovium [37], we demonstrated that serum IL-17 levels were significantly elevated and correlated with disease activity in both AOSD patients and SLE patients. Our results were consistent with the results of recent studies describing an increase in serum IL-17 levels in patients with active SLE [9, 10]. Because IL-17 can stimulate the monocytes to produce pro-inflammatory cytokines including IL-1β and IL-6, thus amplifying the inflammatory cascade [5, 38], these observations suggest an important role for IL-17 in the systemic inflammation of both diseases. However, a large prospective cohort study should be conducted to support our findings.

IL-18 is a pleiotropic and multifunctional pro-inflammatory cytokine that plays an important role in the pathogenesis of AOSD and SLE [18–21, 39, 40]. In accordance with previous studies [18–21, 39, 40], we showed that serum IL-18 levels were significantly elevated in both patients with active AOSD and patients

**TABLE 2** Correlation coefficients (γ) and significant levels of the correlation between circulating Th17 cells frequencies and DAS as well as serum levels of Th17-related cytokines in AOSD patients, SLE patients and healthy controls

<table>
<thead>
<tr>
<th>Variable</th>
<th>γ (AOSD)</th>
<th>γ (SLE)</th>
<th>γ (HC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AOSD activity score</td>
<td>0.527*</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>SLEDAI</td>
<td>–</td>
<td>0.663*</td>
<td>–</td>
</tr>
<tr>
<td>IL-17</td>
<td>0.714***</td>
<td>0.626**</td>
<td>0.573</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.658***</td>
<td>0.719**</td>
<td>0.116</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.493*</td>
<td>0.791***</td>
<td>–0.035</td>
</tr>
<tr>
<td>IL-18</td>
<td>0.621**</td>
<td>0.685*</td>
<td>0.112</td>
</tr>
<tr>
<td>IL-21</td>
<td>0.824***</td>
<td>0.812***</td>
<td>0.490</td>
</tr>
<tr>
<td>IL-23</td>
<td>0.935***</td>
<td>0.756**</td>
<td>0.615*</td>
</tr>
</tbody>
</table>

*P < 0.05, **P < 0.01, ***P < 0.001, determined by the non-parametric Spearman’s rank correlation test. HC: healthy control.

Fig. 2 The levels of serum IL-17 (A), IL-18 (B), IL-1β (C), IL-6 (D), IL-21 (E) and IL-23 (F) were obtained from 24 active untreated patients with AOSD, 16 active patients with SLE and 12 HCs. The horizontal line indicates the median value for each group. *P < 0.001, **P < 0.005, vs healthy controls, determined by Mann–Whitney U-test.
with active SLE. Moreover, a positive correlation between frequencies of circulating Th17 cells and serum IL-18 levels was observed in our patients with AOSD and SLE. Our results support the findings of a recent study showing that Th17 cells in SLE patients may be closely influenced by IL-18 activation [10] and another study showing the role of IL-18 in the induction of Th17 cells [41]. However, the mechanism of IL-18 in perpetuating Th17 cells in autoimmune diseases has not yet been determined.

IL-6 synergizes with IL-1β to enhance the differentiation and the generation of Th17 cells [23]. IL-21 is required to reinforce Th17 cell differentiation [24, 25] and to play a critical role in Th17-dependent autoimmune diseases [25]. In contrast to IL-12, IL-23 does not promote the development of Th1 cells, but is crucial for the expansion and maintenance of Th17 cells [26, 27]. Our results showed that the levels of serum IL-1β, IL-6, IL-21 and IL-23 were significantly elevated and positively correlated with frequency of circulating Th17 cells in both AOSD patients and SLE patients, supporting the role of these cytokines in the differentiation and expansion/maintenance of Th17 cells [22–27, 41].

During a longitudinal follow-up of AOSD patients, we found that the frequency of circulating Th17 cells and the level of serum IL-17 decreased significantly, parallelling the clinical remission and a decrease in inflammatory parameter after effective therapy (Fig. 3). Our results were consistent with the findings of previous animal studies showing that blockade of IL-17 via either mAbs or soluble IL-17 receptor has therapeutic benefits [13, 42]. Phase I trials showed that anti-IL-17 antibodies significantly reduce the signs and symptoms of RA with a decrease in levels of CRP [43–45]. A number of mAb-mediated IL-17 inhibition approaches proceed to Phase III clinical trials for patients with autoimmune diseases.

In conclusion, our results show that the levels of circulating Th17 cells and serum Th17-related cytokines are significantly elevated and positively correlated with disease activity in AOSD. Although the sample size of this study was too small to obtain a definitive conclusion, our findings may provide a clue to understanding the potential roles of Th17 cells and Th17-related cytokines in the immune response of AOSD. The overproduction of Th17-related cytokines may be critically important therapeutic targets in AOSD. However, further study on the pathobiology of Th17 cells in AOSD is needed.

**Rheumatology key messages**

- The frequency of circulating Th17 cells is elevated and correlated with disease activity in AOSD.
- Th17 cells dysregulation may be a pathogenic mechanism for the development of AOSD and SLE.

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**Disclosure statement:** The authors have declared no conflicts of interest.

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