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ORIGINAL ARTICLE

Persistent improper upregulation of Th17 and T_{Reg} cells in patients with juvenile idiopathic arthritis

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Juvenile idiopathic arthritis;
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Objectives: Juvenile idiopathic arthritis (JIA) is the most common childhood rheumatic disease. A break in the balance between Th17 and T_{Reg} cells has been reported as an important factor in the development of autoimmune diseases. This study aimed to analyze peripheral Th17 and T_{Reg} cell levels in patients with JIA.
Methods: The balance of Th17 and T_{Reg} cells among active and inactive JIA patients and normal control subjects were compared. Human peripheral blood mononuclear cells were isolated from the patients and controls. Surface and intracellular staining for CD4, CD25, Foxp3, IL-17, Th17, and T_{Reg} were analyzed.
Results: Twenty-eight JIA patients, including 12 with active JIA and 16 with inactive JIA, and 20 health controls were analyzed. Patients with active JIA had higher Th17 (1.85 ± 1.15 vs. 1.05 ± 0.72, $p = 0.008$) and T_{Reg} cells (1.1 ± 0.8 vs. 0.6 ± 0.7, $p = 0.04$) levels than those with inactive JIA. Among active JIA patients, remission days were highly correlated with the $CD4^+IL17A^+$ T cell percentage, 276.5 ± 137.40 days (range, 130 ~ 525 days), $p < 0.01$. There were no differences in Th17/T_{Reg} percentage between JIA patients and controls in the peripheral blood.
Conclusions: Th17 and T_{Reg} cell levels are elevated in patients with active JIA and there is no Th17/T_{Reg} imbalance. The higher Th17 level predicted longer period to reach disease inactive stage. Improper Th17 up-regulation might contribute to JIA activation.

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Introduction

Juvenile idiopathic arthritis (JIA) is the most common rheumatic disease in childhood and is defined as local inflammation in one or more joints persisting for more than 6 weeks and beginning before the age of 16 years.[1] The reason for this autoimmune activation in JIA is unclear. To date, one of the important mechanisms of this clinically heterogeneous group of chronic pediatric arthritis with unknown etiology is characterized by T cell-dependent inflammation of the synovium, resulting in progressive cartilage destruction, bone damage, and joint destruction.[2]

Naïve $CD4^+$ T cells can differentiate into various subtypes of helper T cells with distinct functions and cytokine profiles. Among the various helper T cell subsets, interleukin (IL)-17-producing helper T (Th17) cells are distinguished from Th1 or Th2 cells by their expression of IL-17A, IL-17F, IL-21, and IL-22.[3] IL-17 is a cytokine participating in tissue inflammation by inducing the expression of proinflammatory cytokines, chemokines, and matrix metalloproteinases.[4] Weaver et al[5] reported that IL-17 drives neutrophil differentiation, monocyte activation, and synovial fibroblast activation, chemokine release, prostaglandin production, and matrix metalloproteinase synthesis, thereby contributing to autoimmunity. Moreover, Th17 cells can cooperate with Th1 cells for interferon-gamma production, further contributing to the proinflammatory state.[6]

At present, numerous studies on mice characterize Th17 as effector cells in various settings, including inflammation, autoimmunity, and immune defense.[4] Enhanced IL-17 expression and increased serum IL-17 levels have been observed in target tissues of patients with various autoimmune diseases, such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA).[7–9] Elevated IL-1, tumor necrosis factor alpha (TNF-α), and IL-6 levels are reported in patients with JIA[10] and a synergistic interaction between IL-17, IL-1, and TNF-α leading to synovial fibroblast activation and cytokine secretion suggests that IL-17 plays a role in the pathogenesis of joint destruction.[11] An excess of Th17 cells has been detected in adults with RA and in children with JIA, particularly in those with more advanced disease.[12]

In contrast to Th17 cells, regulatory T (T_{Reg}) cells, defined by the expressions of CD4, CD25, and the transcription factor forkhead box P3 (FoxP3), have a central role in protecting an individual from autoimmunity.[13] T_{Reg} cells maintain tolerance to self-components by contact-dependent suppression or by releasing anti-inflammatory cytokines like IL-10 and transforming growth factor beta (TGF-β).[14] The suppressive effects of T_{Reg} cells prevent autoimmune flare-ups and chronic inflammation.[15] Dysregulation of T_{Reg} cells are currently considered key factors in several autoimmune diseases such as type 1 diabetes mellitus, multiple sclerosis, inflammatory bowel disease, and psoriasis.[16,17] Some studies suggest that the balance between Th17 and T_{Reg} cells is important in the development/prevention of inflammatory and autoimmune diseases.[16,17] Thus, identifying the detailed relationship between Th17 and T_{Reg} cells and interacting molecules is important for controlling the progression of various diseases.

Although both Th17 cells and T_{Reg} cells contribute to the pathogenesis of autoimmune disease, little is known about their relationship in JIA. Nistala et al[18] have indicated that Th17 cells are highly enriched in the JIA synovium and have a reciprocal relationship with T_{Reg} cells. The present study aimed to analyze the relationship between JIA and Th17/T_{Reg} cells by quantifying $CD4^+$ Th17 and $CD4^+CD25^+FoxP3^+$ T_{Reg} cells in peripheral blood from JIA patients and healthy controls using surface staining, intracellular cytokine staining, and flow cytometry analysis. Because JIA flare-up could lead to severe joint destruction and systemic involvement as the result of increasing autoimmunity, patients with JIA were further divided into active and inactive groups.

Materials and methods

From December 2009 to December 2012, JIA patients on regular follow up at Chang Gung Memorial Hospital, a tertiary care center in Taiwan, were randomized in this cross-sectional study. Mononuclear cells were isolated from paired samples of peripheral blood from patients fulfilling the International League of Associations for Rheumatology criteria for JIA and from healthy individuals, for analysis of Th17 and T_{Reg} cells phenotype.[19] Patients with JIA were categorized into active stage if they had arthritis with or without systemic features and raised C-reactive protein (CRP)/erythrocyte sedimentation rate (ESR).[20] Active disease in the polyarticular and oligoarticular onset groups was defined as at least one joint that had symptoms of active arthritis (swelling, or if swelling was not present, limitation of movement, accompanied by pain or tenderness on motion, or heat). Active disease in the systemic-onset group was defined as at least one joint with active arthritis (as above) or a fever of $>38.5^{\circ}C$ at least 4 days/week without definable infection or other identifiable sources other than JIA. Patients with active systemic-onset juvenile RA (JRA) were further categorized according to the persistence or absence of systemic features, which were defined as the presence of a high spiking fever ($>38.5^{\circ}C$) at the time of sampling. Remission was defined as the absence of active synovitis (morning stiffness not exceeding 15 minutes, no fatigue, no joint pain, no joint tenderness, and no joint or tendon sheath swelling), as well as normal ESRs associated with normal serum CRP concentrations during the previous 3 months.[21,22]

All of the patients were assessed in terms of clinical manifestations, disease activity, and treatment regimens. It is reported that the usage of anti-TNF alpha agents would decrease Th17 cells in peripheral blood.[23] The patients were excluded in this study if they had received anti-TNF alpha agents or biologics in the past 6 months. Age-matched healthy control individuals, who did not have any chronic disease or medication in the previous year, were recruited for comparison. The hospital's ethics committee approved the study (IRB number: 100-4299A3) and all of the study participants and their parents provided informed consent.

Collection and preparation of blood samples

Blood specimens were drawn following an overnight fast for baseline evaluation. Mononuclear cells were isolated from

patients with JIA ($n = 28$) and healthy individuals ($n = 20$). Blood specimens were placed into collection tubes containing 0.2 mL sodium heparin. Peripheral blood mononuclear cells (PBMCs) were prepared by Ficoll density gradient for analysis using flow cytometry.

Cell preparation

For analysis of Th17 cells, PBMCs were suspended at a density of 2×10^6 cells/mL in complete culture medium (RPMI 1640 supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM glutamine, and 10% heat-inactivated fetal calf serum, Burlington, Ontario, Canada, Gibco BRL). The cell suspension was transferred to 24-well plates and cultures were stimulated using phorbol myristate acetate (50 ng/mL) plus ionomycin (1 μM) for 4 hours in the presence of monensin (500 ng/mL, all from Alexis Biochemicals, San Diego, CA, USA) The incubator was set at 37°C under 5% CO_2 environment. After 4 hours of culture, the contents of the wells were transferred to 5-mL sterile tubes. The cells were then centrifuged at 1500 rpm for 5 minutes.

Surface and intracellular staining for Th17 and T_{Reg} cells

The cells were stained with fluorochrome-conjugated monoclonal antibodies and their appropriate isotype controls. Cells were aliquoted into tubes and washed once in phosphate-buffered saline. For Th17 analysis, the cells were incubated with phycoerythrin (PE) antihuman CD4 (eBioscience, San Diego, CA, USA) at 4°C for 20 minutes. For T_{Reg} analysis, the cells were incubated with peridinin chlorophyll antihuman CD4 and fluorescein isothiocyanate (FITC) antihuman CD25. After the surface staining, the cells were stained with FITC antihuman IL-17A for Th17 detection or PE antihuman FoxP3 for T_{Reg} detection after fixation and permeabilization (BD Cytofix/Cytoperm, San Jose, CA, USA) according to the manufacturer's instructions. Isotype controls were given to enable correct compensation and confirm antibody specificity. All of the antibodies were from eBioscience. Stained cells were analyzed by flow cytometric analysis using a FACS (Fluorescence-Activated Cell Sorting) cytometer (BD FACSCanto II Flow Cytometry System, San Jose, CA, USA) equipped with CellQuest software (BD Bioscience PharMingen, San Jose, CA, USA). An isotype-matched control monoclonal antibody was used to determine nonspecific staining. Expression of cell surface or intracellular proteins was quantified using flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA, USA) and data were analyzed using the CellQuest Pro software (BD Biosciences). The purity of isolated cells, as assessed by flow cytometry using anti-CD3, anti-CD4, anti-CD25, anti-IL17A, and anti-FoxP3 antibodies, was >85%.

Statistical analysis

Comparisons of the Th17 and T_{Reg} cell percentages and Th17/T_{Reg} ratio were made between the JIA and control groups. Following international criteria, JIA patients were divided into active and inactive groups for further evaluation.[20] For all major results, 95% confidence intervals (CIs) were given. All comparisons between JIA patients and controls were made using Student t test. Statistical significance was set at $p \leq 0.05$.

Results

Twenty-eight patients with JIA and 20 healthy controls completed the T_{Reg}/Th17 phenotype analysis. The mean age of JIA patients and the controls was 18.2 ± 6.4 years (range, 7.72–30.41 years) and 19.21 ± 4.22 years (range, 11.09–30.67 years), respectively. There was no significant difference in age and follow-up duration between these two groups.

Of the 28 JIA patients, 14 were oligoarticular type, 10 were polyarticular type, and four were systemic type. Thirteen were male and 15 were female. Twelve patients had active disease and 16 had inactive disease. Their characteristics and current medication are shown in Table 1. Among 12 active JIA patients, all became disease inactive stage during the follow-up period. The mean remission duration of active JIA patients was 276.5 ± 137.40 days (range, 130∼525 days).

The mean $CD4^+$ T cell percentage was 40.0 ± 8.5% in JIA patients and 40.8 ± 6.7% in healthy individuals. The mean $CD4^+CD3^+CD25^+$ T cell percentage in patients with JIA was 9.2 ± 4.6% (range, 2.8–14.8%) and the mean $CD4^+CD25^+FoxP3^+$ T cell percentage was 0.9 ± 0.8% (range, 0–2.4%). Their mean $CD4^+CD3^+IL17A^+$ T cell percentage was 1.52 ± 0.88% (range, 0–3.6%) and their mean Th17/T_{Reg} ratio was 1.71 ± 1.0.

Among the controls, the mean $CD4^+CD25^+FoxP3^+$ T cell percentage was 0.8 ± 0.5% (range, 0–2.0%). The mean $CD4^+CD3^+IL17A^+$ T cell percentage was 1.4 ± 1.0% (range, 0–2.7%) and the mean Th17/T_{Reg} ratio was 1.75 ± 1.0.

Table 1 Clinical characteristics of patients with juvenile idiopathic arthritis ($n = 28$)

	Active JIA ($n = 12$)	Inactive JIA ($n = 16$)
Characteristics		
Age (y)	18.5 ± 6.54	18.19 ± 6.59
Range (y)	6.87–30.65	8.83–30.12
Duration of disease (y)	8.5 ± 4.56	8.7 ± 5.14
Sex (male/female)	6 vs. 6	7 vs. 9
Systemic type ($n = 4$)	1	3
Oligoarticular type ($n = 14$)	5	7
Polyarticular type ($n = 12$)	6	6
Time to remission (d)	276.5 ± 137.40	
Remission range (d)	130–525	
Current treatment of sample (number/%)		
MTX	3	0
NSAIDs	11	6
Prednisolone	2	0
None	0	10

JIA = juvenile idiopathic arthritis; MTX = methotrexate; NSAID = nonsteroidal anti-inflammatory drug.

Table 2 Frequencies of surface and intracellular staining of circulating T cells

Cell marker	Controls	JIA	Active JIA	Inactive JIA
	$n = 20$	$n = 28$	$n = 12$	$n = 16$
$CD4^+$	40.8 ± 6.7	40.0 ± 8.5	40.8 ± 7.9	39.9 ± 9.1
$CD4^+CD3^+CD25^+$	8.1 ± 1.8	9.2 ± 4.6	9.2 ± 5.0	9.0 ± 4.3
$CD4^+CD3^+IL17\ A^+$	1.4 ± 1.0	1.52 ± 0.88	1.85 ± 1.15	1.05 ± 0.72
$CD4^+CD25^+FoxP3^+$	0.8 ± 0.5	0.9 ± 0.8	1.1 ± 0.8	0.6 ± 0.7
$Th17/T_{Reg}$	1.75 ± 1.0	1.71 ± 1.1	1.68 ± 1.0	1.81 ± 1.2
p	Controls vs. JIA	Active JIA vs. inactive JIA	Inactive JIA vs. NC	Active JIA vs. NC
$CD4^+$	0.92	0.83	0.88	0.94
$CD4^+CD3^+CD25^+$	0.2	0.49	0.43	0.5
$CD4^+CD3^+IL17A^+$	0.46	0.008*	0.04*	0.25
$CD4^+CD25^+FoxP3^+$	0.48	0.04*	0.34	0.29
$Th17/T_{Reg}$	0.27	0.15	0.24	0.54

*$p < 0.05$.
Active juvenile idiopathic arthritis (JIA) patients had higher Th17 and T_{Reg} cell levels than inactive JIA patients ($p = 0.008$ and 0.04, respectively); controls had higher levels of Th17 cells than patients with inactive JIA ($p = 0.04$).

There was no difference in the $CD4^+CD25^+$ T cell ratio between the JIA and the control groups. In analysis of $CD4^+CD25^+FoxP3^+$ T cells (T_{Reg}) and $CD4^+IL17A^+$ T cells (Th17), there was also no significant difference observed. There was no significant difference in $Th17/T_{Reg}$ ratio between patients with JIA and healthy controls ($p = 0.27$; Table 2).

T_{Reg} cells and Th17 cells in active and inactive JIA

To analyze whether disease activity could influence $Th17/T_{Reg}$ balance, the 28 JIA patients were divided into active and inactive JIA groups. The mean $CD4^+CD25^+$ T cell percentage in the active JIA and inactive JIA groups was $9.2 \pm 5.0\%$ and $9.0 \pm 4.3\%$ ($p = 0.49$), respectively, and the mean $CD4^+IL17A^+$ T cell percentage was $1.85 \pm 1.15\%$ and $1.05 \pm 0.72\%$ ($p = 0.008$), respectively (Fig. 1A). The mean $CD4^+CD25^+FoxP3^+$ T cell percentage was $1.1 \pm 0.8\%$ and $0.6 \pm 0.7\%$ ($p = 0.04$), respectively (Fig. 1B) and the $Th17/T_{Reg}$ ratio was 1.68 ± 1.0 and 1.81 ± 1.2 ($p = 0.15$), respectively. Taken together, in active JIA patients, the percentages of T_{Reg} and Th17 cells were both higher in the peripheral blood, but the $Th17/T_{Reg}$ ratio did not have any significant difference. By dividing the JIA patients into active and inactive stages, the representative flow cytometry figures for Th17 and T_{Reg} cells are shown in Fig. 2A and B, respectively. Among 12 active JIA patients, all became disease inactive stage during the follow-up period. From the days of enrollment, among active JIA patients, remission days were highly correlated with the $CD4^+IL17A^+$ T cell percentage, 276.5 ± 137.40 days (range, 130—525 days), $p < 0.01$ (Fig. 3). The percentage of T_{Reg} and the $Th17/T_{Reg}$ ratio did not have any significant difference in remission duration.

Further analysis was made between patients with active JIA and the healthy controls. There were no significant differences in serum Th17 and T_{Reg} cell percentages. Thus, JIA patients might have the same autoimmune status as the healthy population during the disease inactive stage. Further comparisons made between patients with inactive JIA and the healthy controls revealed that patients with inactive JIA had significantly lower $CD4^+IL17A^+$ T cell percentages ($p = 0.04$), indicating that inactive JIA patients had significantly lower Th17 cells than the controls (Table 2).

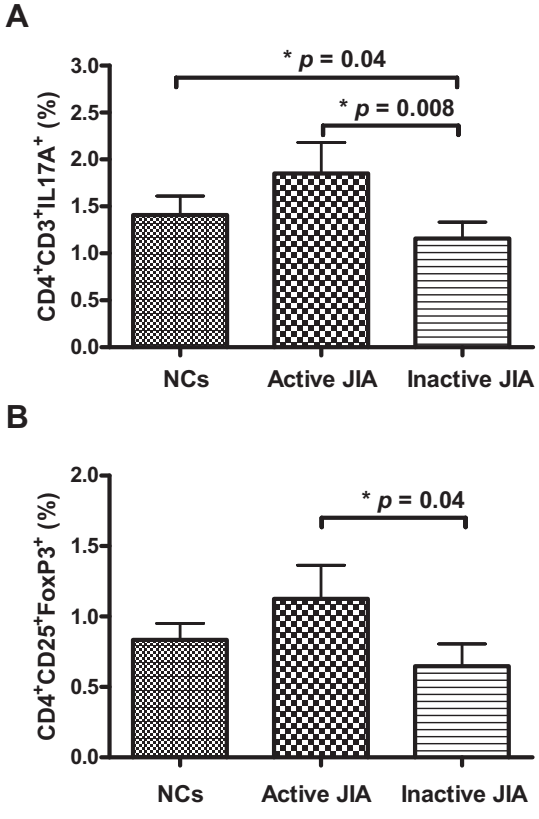

Figure 1. (A) Patients with active juvenile idiopathic arthritis (JIA) had higher levels of both Th17 and T_{Reg} cells than inactive JIA patients ($p = 0.008$ and 0.04, respectively); (B) controls had higher levels of Th17 cells compared to patients with inactive JIA ($p = 0.04$).

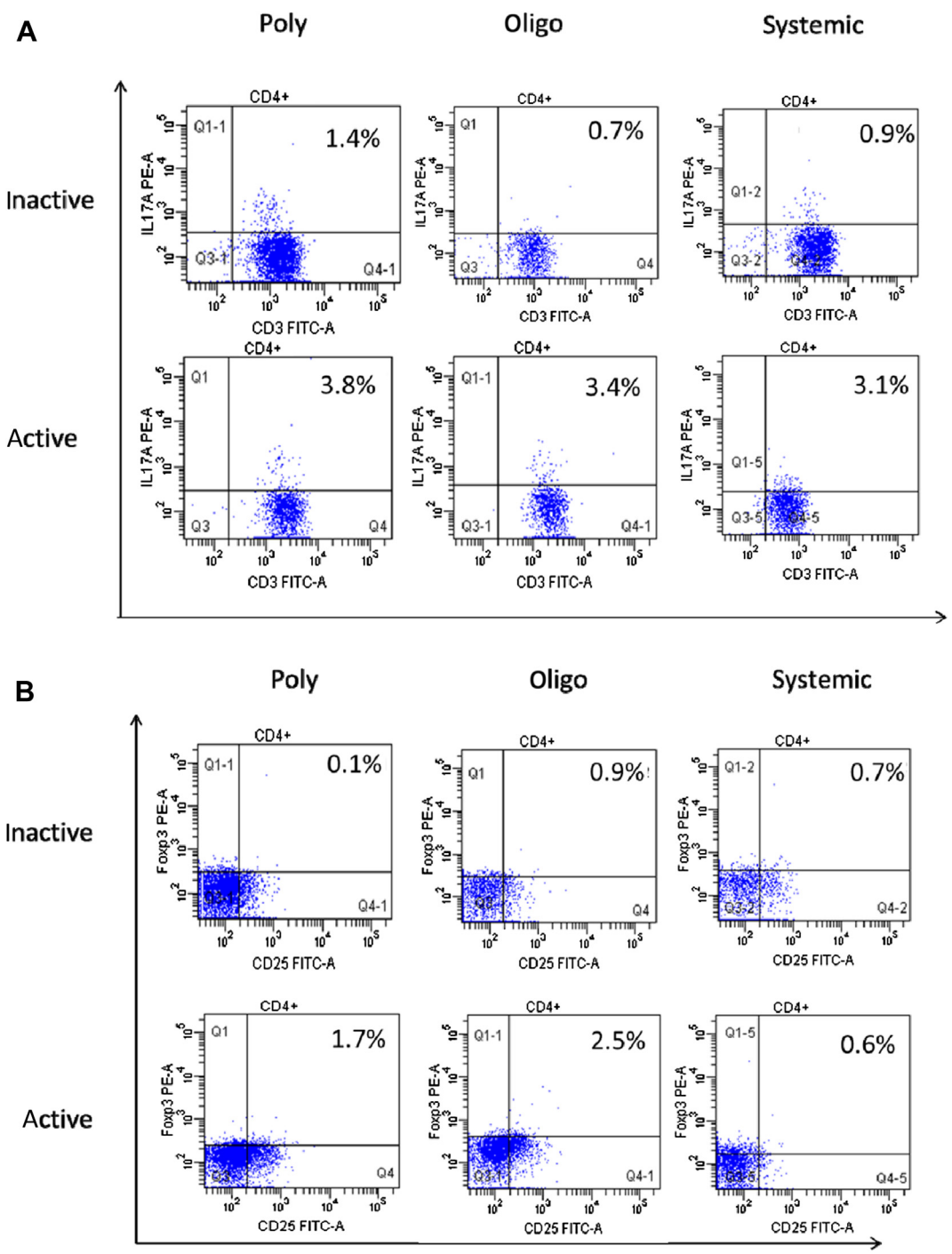

Figure 2. Representative flow cytometry figures for Th17 and T_{Reg} cells in three subtypes of JIA. (A) Active juvenile idiopathic arthritis (JIA) patients had higher levels of peripheral Th17 cells than inactive JIA patients; (B) active JIA patients had higher levels of peripheral T_{Reg} cells than inactive JIA patients.

Discussion

This study attempted to analyze the association between Th17-T_{Reg} phenotype and JIA patients. There were higher levels of both Th17 and T_{Reg} cells in active JIA patients than inactive JIA patients (both $p < 0.05$). The time taken to reach the remission stage was highly correlated with the Th17 level in active JIA patients ($p < 0.05$). There was no significant difference in the ratio of Th17/T_{Reg} between the two groups.

IL-17 can stimulate fibroblasts to produce IL-6 and IL-8, which are both inflammatory cytokines.[24] By synergistic interactions among IL-17, IL-1, and TNF-α, Th17 plays a major role in synovial fibroblast activation and joint destruction by cytokine secretion.[11] Recent studies have viewed IL-17-producing Th17 cells as important T cells in

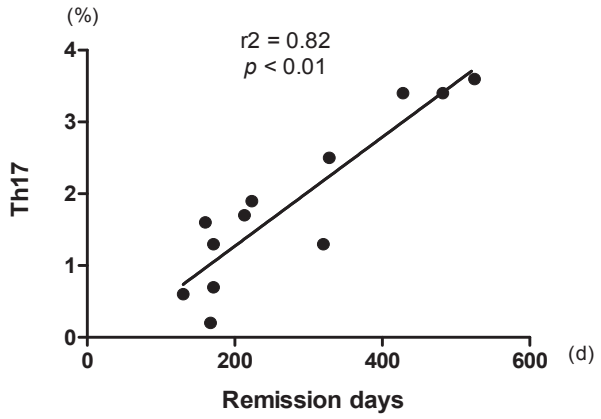

Figure 3. From the days of enrollment, among active juvenile idiopathic arthritis (JIA) patients, remission days were highly correlated with the $CD4^+IL17A^+$ T cell percentage, 276.5 ± 137.40 days (range, 130–525 days), $p < 0.01$.

autoimmune inflammation.[9] Higher synovial fluid IL-17 levels are observed in patients with RA, whose enhanced IL-6 production results in bone and collagen destruction.[25,26] Improper Th17 accumulation is closely related to autoimmune disease development. Yang et al[27] reported that disease activity closely correlated with serum Th17 levels. In the present study, the $CD4^+IL17A^+$ level is significantly higher in patients with active JIA compared to those with inactive disease ($p = 0.008$). The duration to reach remission stage in active JIA patients was highly correlated with the peripheral Th17 level in this study—276.5 ± 137.40 days (range, 130–525 days), $p < 0.001$. Thus, the disease status may exert influence in the proinflammatory-Th17 pathway and poorly controlled disease activity may result in unduly elevated Th17 cells, which may be one mechanism that results in disease progression. This observation is corroborated by lower Th17 cell activity and peripheral Th17 cells in JIA patients with inactive disease. At present, dysregulation of T_{Reg} cells is considered a key factor in several autoimmune diseases, including type 1 diabetes mellitus, multiple sclerosis, SLE, and RA.[16,17] By releasing IL-10 and TGF-β, T_{Reg} cells can maintain tolerance to self-components.[14] Most studies suggest that T_{Reg} cells are essential for the control of self-tolerance, based on the fact that depletion of naturally occurring T_{Reg} cells results in hyperproliferation of effector T cells and autoimmunity.[28,29] However, plenty of studies have documented that T_{Reg} cells accumulate in the synovial fluid and peripheral blood of patients with RA and JIA.[30,31] Nonetheless, inflammation progresses.

Baecher-Allan et al[32] indicated that preactivated responder $CD4^+$ T cells become resistant to regulation by $CD4^+CD25^+$ T cells *in vitro*. This resistance is dependent on the strength and duration of the stimulus. The present study has shown that patients with active JIA have much higher levels of T_{Reg} cells in peripheral blood than those with inactive JIA. There is also no significant difference in T_{Reg} cell concentration between healthy controls and active JIA patients. Thus, the increase in $CD4^+CD25^+$ $FoxP3^+$ T_{Reg} cells in active JIA patients may be due to the increasing resistance of effector T cells to the suppression effects of T_{Reg}.

Some studies have indicated that during the inactive stage, JIA patients do not have any symptoms and have the same immune profile.[17] In the present study, none of the16 inactive JIA patients used steroids and only six received nonsteroidal anti-inflammatory drugs during the enrollment period (Table 1). There were significantly fewer Th17 cells in inactive JIA patients than in healthy controls (1.05 ± 0.72 vs. 1.4 ± 1.0) and a lower T_{Reg} cell concentration was observed in inactive JIA patients compared to controls. A previous study found that the use of immunosuppressants may suppress immune flare-ups, especially when steroids with long-lasting and cumulative effects are used.[33] Thus, the much lower concentration of Th17 cells in inactive JIA patients compared to healthy controls may be attributed to the use of immunosuppressants. Further evaluation of cumulative steroid dosage among JIA patients revealed no significant association (data not shown).

Recent evidence indicates that both Th17 cells and natural T_{Reg} cells contribute to the pathogenesis of autoimmune disease.[6–13] Some studies even suggest that the balance between Th17 and T_{Reg} cells is the most important factor in the development/prevention of inflammatory and autoimmune diseases.[16,17] However, through the present study, no Th17/T_{Reg} imbalance was observed ($p = 0.27$). Instead of T_{Reg} cell depletion, both Th17 and T_{Reg} upregulation were observed in active JIA patients (both $p < 0.05$). T_{Reg} cells act as inflammation protectors and many studies suggest their decrease during disease flare-up. However, there are many existing materials that indicated improper T_{Reg} cell accumulation in the synovial cavity and blood.[29–31] Taken together, Th17 activation seems to have the dominant role in JIA activation and the upregulation of T_{Reg} cells may be due to increased resistance to effector T cells.

This study has several limitations. First, patients with a history of JIA were enrolled but no newly-diagnosed JIA and drug-sparing patients were included to identify drug effects. Instead, cumulative steroid dose was calculated for analysis and there was no positive association. Second, cytokine evaluation to determine if JIA patients have higher resistance to T_{Reg} cell suppression was not carried out. Lastly, the study involved only a small group of JIA patients, particularly when considering the active versus inactive groups and the immunosuppressant medication subgroups.

Patients with active JIA have elevated levels of Th17 and T_{Reg} cells. The higher Th17 level predicted a longer period to reach disease inactive stage. There is no Th17/T_{Reg} imbalance so improper Th17 upregulation may contribute to the activation of JIA.

Conflicts of interest

The authors declare that they have no competing interests.

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