Th1 and Th17 Predominance in the Enthesitis-related Arthritis Form of Juvenile Idiopathic Arthritis

ANKIT MAHENDRA, RAMNATH MISRA, and AMITA AGGARWAL

ABSTRACT. ***Objective.*** A Th1 biased immune response in synovial fluid has been reported in children with polyarticular and extended oligoarticular-type juvenile idiopathic arthritis (JIA). We investigated T cell phenotypes including Th1, Th2, Th17, and Treg with emphasis on Th17 and Treg, in order to differentiate cytokines in the enthesitis-related arthritis (ERA) form of JIA.

Methods. The frequencies of Th1, Th2, Th17, and Treg cells were determined by flow cytometry in peripheral blood (PB) and synovial fluid from patients with ERA and healthy subjects. Levels of interleukin 1ß (IL-1ß), IL-6, IL-21, IL-23, and transforming growth factor ß (TGF-ß), cytokines that influence Th17 lineage cells, were measured in paired plasma and synovial fluid (SF) samples by ELISA. Frequencies are expressed as percentages and cytokine levels as pg/ml.

Results. There were no differences in blood samples in the frequency of Th1, Th2, Th17, and Treg cells between patients and controls. In paired samples, the median frequency of CD4+IFN-γ+ (20.49 vs 4.03; $p < 0.005$) and CD4+IL-17+ (2.27 vs 0.57; $p < 0.01$) cells was significantly higher in SF compared to PB, respectively; whereas the frequency of CD4+IL-4+ (1.79 vs 2.29; $p < 0.04$) cells was significantly reduced in the SF compared to PB. There was no difference in the frequency of regulatory T cells. Patients receiving methotrexate had fewer Th2 cells, whereas the Childhood Health Assessment Questionnaire score had a negative association with the frequency of Treg. Median levels of IL-1ß ($p < 0.008$), IL-6 ($p < 0.0001$), and IL-17 ($p < 0.0001$) were higher in SF than in plasma and levels of TGF-ß were lower ($p < 0.001$). Levels of IL-21 were similar in SF and plasma, whereas IL-23 was undetectable.

Conclusion. In patients with ERA, peripheral blood Th1, Th2, Th17, and Treg cells were unchanged, but Th1 and Th17 cells were increased and Th2 cells were reduced in the SF compared to blood. Elevated IL-1ß and IL-6 in SF may be responsible for increased Th17 cells. (J Rheumatol First Release June 15 2009; doi:10.3899/jrheum.081179)

Key Indexing Terms:
JUVENILE IDIOPATHIC ARTHRITIS CYTOKINES T CELL PHENOTYPE
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In inflammatory joint diseases the synovial membrane is rich in activated T cells, suggesting that these cells are key agents in the pathogenesis of these diseases[1]. T cells can be categorized as proinflammatory [T helper-1 (Th1) or T helper-17 (Th17)] or antiinflammatory [T helper-2 (Th2) or T regulatory (Treg)]. In rheumatoid arthritis (RA), Th1 are the major cells in synovial tissue and synovial fluid (SF)[2,3]. In juvenile idiopathic arthritis (JIA), levels of interferon-γ (IFN-γ), a cytokine produced by Th1 cells, are increased in both serum and SF[4]. Th1 cells activate macrophages and fibroblasts in the synovial tissue, leading to production of mediators of tissue destruction[5].

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Th17 cells, a subset of IL-17-producing T cells, have been shown to play an important role in the pathogenesis of autoimmune diseases. Expression of IL-17 is increased in the synovium and SF fluid of patients with RA[6] and JIA[7]. IL-17-knockout mice and mice that have been administered IL-17 receptor IgG1 Fc fusion protein are partly protected against the development of arthritis[8,9]. IL-17 produced by Th17 cells induces production of proinflammatory cytokines like IL-6, IL-8, IL-1, and tumor necrosis factor-α, thus perpetuating inflammation.

Th2 and Treg cells have antiinflammatory properties. In an animal model of RA, Th2 cells and cytokines produced by these cells such as IL-10 and IL-4 strongly suppressed tissue inflammation and tissue destruction. Administration of IL-10 or IL-4 has been shown to result in clinical benefit in patients with RA. Also in patients with RA, fewer IL-4-secreting T cells are present in synovial tissue and SF than IFN-γ-producing cells[10].

Treg are naturally-occurring, thymus-derived T cells that suppress effector T cell function. Treg are characterized by high expression of CD25 and FoxP3 transcription factor. Treg are present in the inflamed synovial tissue and may suppress

autoimmune disease either by cell-cell contact or by secretion of soluble mediators[11].

Generation of different types of T cells from naive T cells depends on the local milieu. Whereas transforming growth factor ß (TGF-ß) is crucial for the development of Treg, IL-1ß, IL-6, IL-21, IL-23, and TGF-ß are important for the generation and maintenance of Th17 cells. Similarly, IL-12 is important for Th1 development and IL-4 for Th2 development.

Studies in JIA have shown a Th1 bias in the synovial compartment, with evidence of high concentrations of IFN-γ in synovial tissue and SF, increased frequency of T cells that bear Th-1-specific chemokine receptors CCR5 and CXCR3, and increased numbers of CD4+IFN-γ+ T cells[12,13]. SF in patients with oligoarticular JIA shows an increased number of CD25hi Treg cells as well as presence of CCR4+ T cells with a Th2 bias, suggesting the presence of antiinflammatory T cells[14,15]. A recent study has shown enrichment of Th17 cells in the SF of children with JIA[7].

JIA is a heterogeneous disease. In Caucasian populations about 3%–11% of patients with JIA have the enthesitis-related arthritis (ERA) subtype. ERA affects boys over the age of 6 years and presents with lower limb oligoarthritis and enthesitis. About 60%–65% of those affected are HLA-B27-positive and some have a family history of spondyloarthropathy. In this subtype, the T cell phenotype of cells in the synovial compartment has not been studied in detail. We have previously reported increased numbers of CCR5+ and CXCR3+ CD4 T cells and elevated levels of CXCL10 in SF of patients with ERA, suggesting a Th1 bias[16]. We also found elevated levels of IL-1ß, IL-6, IL-12, and IFN-γ in SF of patients with ERA[17]. Recently we reported elevated levels of IL-17 in SF of patients with JIA including ERA[18].

As there are few data available on Th17, Treg, and Th2 cells in ERA, we investigated the frequency of Th17, Th1, Th2, and Treg cells in peripheral blood and simultaneously obtained SF of patients with ERA using flow cytometry. Cytokines involved in Th17 and Treg differentiation and maintenance were measured in plasma and SF.

MATERIALS AND METHODS

Consecutive patients with the ERA subtype of JIA diagnosed using the ILAR classification criteria[19] attending the outpatient clinic of our institution and requiring intraarticular injection were recruited for the study. Peripheral blood was collected into a heparinized vial from each patient. Where possible, SF was also collected at the time of intraarticular corticosteroid injection.

All the 19 patients were male. The median age was 15 years (range 9–24) and disease duration 2 years (range 0.25–11). Six patients were receiving methotrexate (MTX), 9 nonsteroidal antiinflammatory drugs, and 4 were not receiving any drug. HLA-B27 was positive in 9 of 10 patients in whom it had been tested. Median number of swollen joints was 1 (range 0–4), tender joints 2 (range 0–6), and joints with limited mobility 2 (range 0–6). Median Childhood Health Assessment Questionnaire (CHAQ) score (scale 0–3), available in 12 patients, was 0.81 (range 0–2.25). SF was available from 16 patients, and sufficient SF mononuclear cells were available from 12 patients.

Twelve subjects attending our hospital with minor complaints but found to have no significant disease were included as controls (median age 10 yrs, range 6–21; 10 boys), and each of them provided a blood sample.

The study was approved by an institutional ethics committee, and all subjects provided written informed consent.

Flow cytometric analysis of T cell phenotypes. SF mononuclear cells were separated by density-gradient centrifugation using Lymphoprep (Sigma, St. Louis, MO, USA) and suspended in RPMI medium (Sigma). SF mononuclear cells and whole blood were activated with 25 ng/ml phorbol myristate acetate (PMA; Sigma) and 1 μg/ml ionomycin (Sigma) for 6 h. The mixture also contained 10 μg/ml Brefeldin A (Sigma) to prevent extracellular secretion of cytokines. Cells were then stained with FITC-labeled monoclonal anti-CD4 antibodies (Becton Dickinson, San Diego, CA, USA). In whole blood, red cells were lysed using FACS lysing solution (Becton Dickinson). After washing with phosphate buffered saline (PBS), the cells were fixed using a fixation buffer (eBiosciences, San Diego, CA, USA) and washed again. The cells were then permeabilized (permeabilization solution; eBiosciences) and incubated with phycoerythrin (PE)-labeled monoclonal antibodies (eBiosciences) against signature cytokines, i.e., IFN-γ (Th1), IL-4 (Th2) and IL-17 (Th17), washed again, and resuspended in PBS.

For identification of Treg, cells were surface-stained with FITC-labeled anti-CD4 and PE-Cy5-labeled anti-CD25 antibodies (eBiosciences), followed by staining for FoxP3 using the FoxP3 staining kit (eBiosciences), following the manufacturer's protocol.

In each assay, markers were drawn based on data from control experiments that used isotype-specific IgG antibodies. Data were acquired using the BD FACSCalibur system and CellQuest Pro software (both Becton Dickinson). Using a lymphocyte gate, 10,000 events were acquired for Th1, Th2, and Th17, and 50,000 events for Treg cells.

Enzyme immunoassays. Concentrations of cytokines in plasma and SF were measured using commercial kits (TGF-ß: BD Biosciences; others: eBiosciences). Minimum detection limits of these assays were 4 pg/ml for IL-17 and IL-1ß, 30 pg/ml for IL-21, 15 pg/ml for IL-23, 2 pg/ml for IL-6, and 30 pg/ml for TGF-ß.

Statistical analysis. Mann-Whitney U test and Wilcoxon's signed-rank test were used for comparison of unpaired and paired data, respectively.

RESULTS

Flow cytometry. The frequencies of Th1, Th2, Th17, and Treg cells in peripheral blood were similar in patients and controls (Table 1, Figure 1).

Comparison of T cell phenotype frequencies in paired blood and SF specimens (Figure 1) revealed that SF had more Th1 cells [median 20.49 (range 10.68–30.23)] than blood [median 4.03 (range 2.22–7.45)] ($p < 0.002$), and fewer Th2 cells [median 1.79 (range 0.44–4.86) vs median 2.29 (range 1.23–4.42)] ($p < 0.041$). The frequency of Th17 cells was also significantly higher in SF [median 2.27 (range 0.77–5.53)] than in blood [median 0.57 (range 0.24–2.71)] ($p < 0.01$). The Treg frequencies were similar in SF [median 1.68 (range 0.11–8.86)] and blood [median 1.16 (range 0.19–7.56)]

Table 1. Proportion of different types of T cells in peripheral blood of patients and controls. All data are percentage of CD cells, median (range).

T Cell Phenotype	Controls	Patients	p
Th1	4.10 (2.04–7.51)	5.59 (2.22–10.52)	NS
Th2	2.36 (0.82–4.25)	2.66 (1.23–5.77)	NS
Th17	1.03 (0.71–1.4)	1.11 (0.24–4.56)	NS
Treg	0.73 (0.30–7.13)	1.16 (0.19–7.56)	NS

NS: nonsignificant.

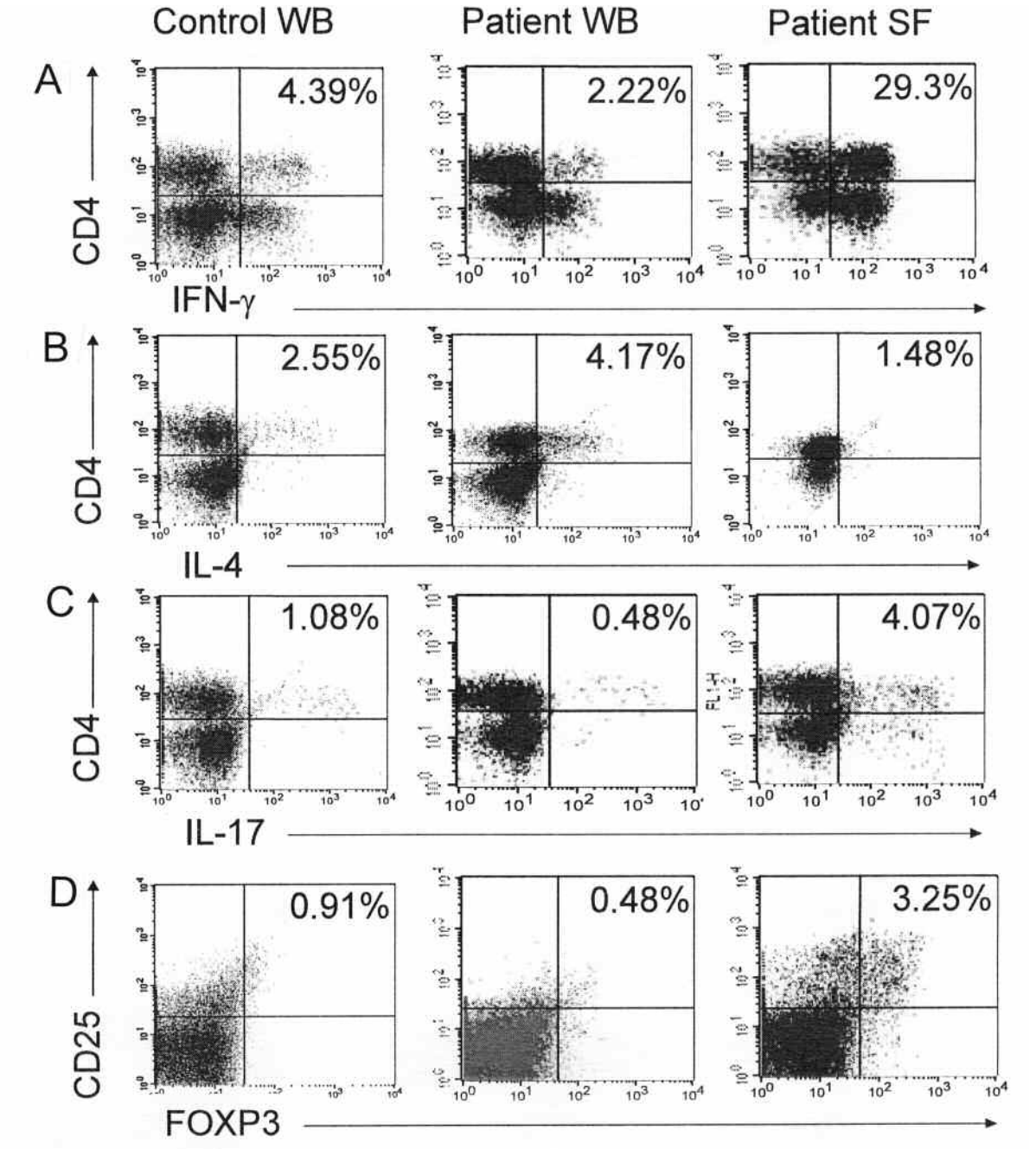

Figure 1. Dot plots show T cell phenotypes in whole blood (WB) from healthy controls and patients with enthesitis-related arthritis, and synovial fluid (SF) mononuclear cells from patients. Proportions of Th1, Th2, and Th17 in the lymphocyte gate were analyzed. CD25+, FoxP3+ cells were analyzed within the CD4 gate. A. Frequencies of Th1 (CD4+, IFN-γ+) cells. B. Frequencies of Th2 (CD4+, IL-4+) cells. C. Frequencies of Th17 (CD4+, IL-17+) cells. D. Frequencies of Treg (CD25+, FoxP3+) cells.

(Figure 2). The proportion of SF Th17 cells varied inversely with that of SF Treg cells (r = –0.537, p = 0.044).

Correlation with disease variables. Patients receiving MTX had lower frequencies of Th2 cells than those not receiving MTX in both blood [median 1.76 (range 1.23–5.07) vs median 3.01 (range 2.08–5.12)] (p = 0.036) and SF [median 1.20 (range 0.90–1.41) vs median 1.96 (range 1.48–4.10)] (p = 0.04). CHAQ scores correlated negatively with the frequency of Treg cells in both blood (r = –0.729, p = 0.007) and SF (r = 0.720, p = 0.029). The number of joints with limited mobility showed good correlation with the frequency of Th1 cells in blood (r = 0.562, p = 0.019), frequency of Th17 cells in blood (r = 0.654, p = 0.004), and frequency of Th17 cells in SF (r = 0.655, p = 0.04).

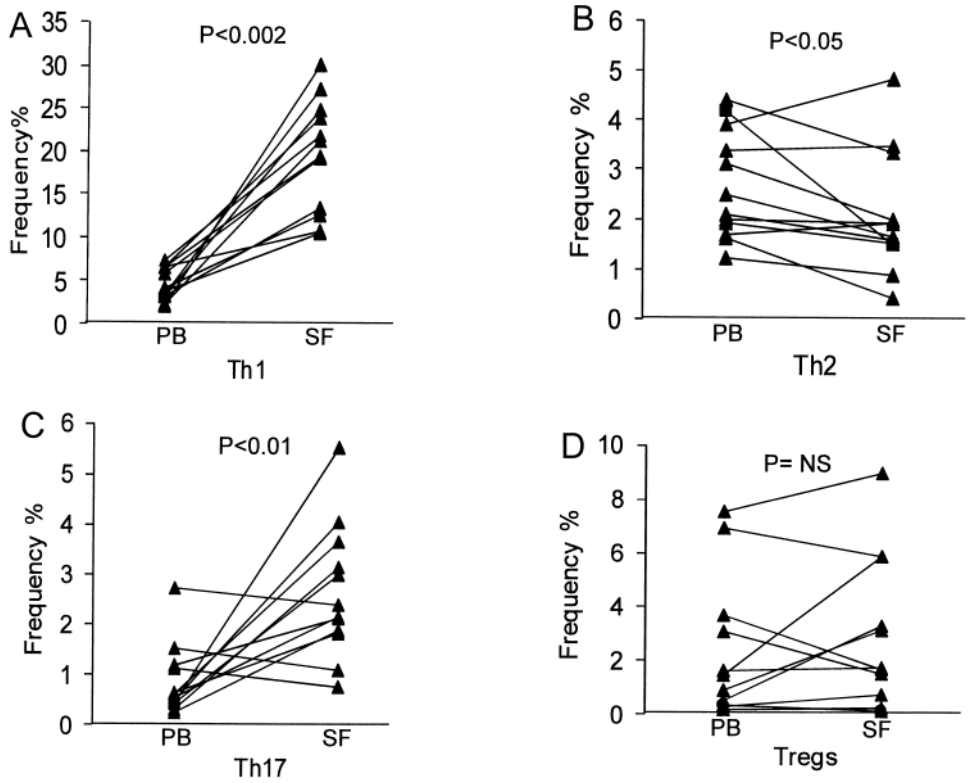

Figure 2. T cell phenotype in simultaneous peripheral blood (PB) and synovial fluid (SF) specimens from patients with enthesitis-related arthritis. A. Frequencies of Th1 (CD4+, IFN-γ+) cells. B. Frequencies of Th2 (CD4+, IL-4+) cells. C. Frequencies of Th17 (CD4+, IL-17+) cells. D. Frequencies of Treg (CD25+, FoxP3+) cells. NS: nonsignificant.

Cytokine levels. Among 16 patients in whom cytokines were measured in both blood and SF, median levels of IL-17, IL-1ß, and IL-6 were higher in SF than in plasma, whereas level of TGF-ß was lower in SF (Table 2, Figure 3). IL-23 was undetectable in both plasma and SF in all subjects.

DISCUSSION

Our data show a higher frequency of Th1 and Th17 cells in the SF and a reduced frequency of Th2 cells, compared to frequency in blood. The frequency of Treg cells was similar in blood and SF. In addition, levels of IL-1ß, IL-6, and IL-17 were higher whereas those of TGF-ß were lower in the SF than in blood. There was no difference in the levels of IL-21, whereas IL-23 was undetectable in both plasma and SF.

T cells play a major role in the pathogenesis of arthritis. Studies have shown a Th1 bias in SF in patients with RA and JIA[3,12,13]. Other indirect evidence of Th1 bias in the synovial compartment includes chemokine receptor analysis and data on mRNA and protein levels of signature cytokines such as IFN-γ[13,16,20]. Our data support these studies. However, in oligoarticular disease, a Th2 bias was observed in SF[15]. Our study is the first to show a Th1 bias in the SF of patients with the ERA type of JIA. The lack of any difference in peripheral blood T cell phenotypes between our patients and controls suggests that immunoinflammation in patients with ERA is localized to the synovial compartment. The excess of Th1 cells in SF that we observed in patients with ERA could be due either to preferential homing of these cells to the synovium or to Th1 differentiation at this site. Our previous work suggests that selective homing mediated by chemokines such as IFN-inducible protein-10/CXCL10 may be responsible for this[16].

Th2 cells and their cytokines have an antiinflammatory effect and thus may play a role in amelioration of inflammation. Patients with persistent oligoarticular disease had more Th2 cells than patients with extended oligoarticular disease[15]. Other studies have shown a paucity of IL-4-secreting cells in both JIA and RA[12,20]. Our study supports these findings. Since Th1 cells inhibit the Th2 cells, and vice versa, the low frequency of Th2 cells that we observed could be related to an increase in the number of Th1 cells. A lower frequency of Th2 cells in patients receiving MTX could be related either to an

Table 2. Cytokine concentrations in plasma and synovial fluid of patients with enthesitis-related arthritis (n = 16). All data are median (range).

Cytokine	Plasma, pg/ml	SF, pg/ml	p
IL-17	< 4.0 (<4–12.8)	43.0 (11.6–1220)	0.0001
IL-1ß	< 4–10.4)	8.4 (< 4.0–3400)	0.008
IL-6	< 2.0 (< 2–180)	10,500 (1700–19,000)	0.0001
IL-21	230 (62–1080)	340 (176–10,000)	0.112
TGF-ß	22,200 (2760–45,600)	2280 (< 30–5280)	0.001

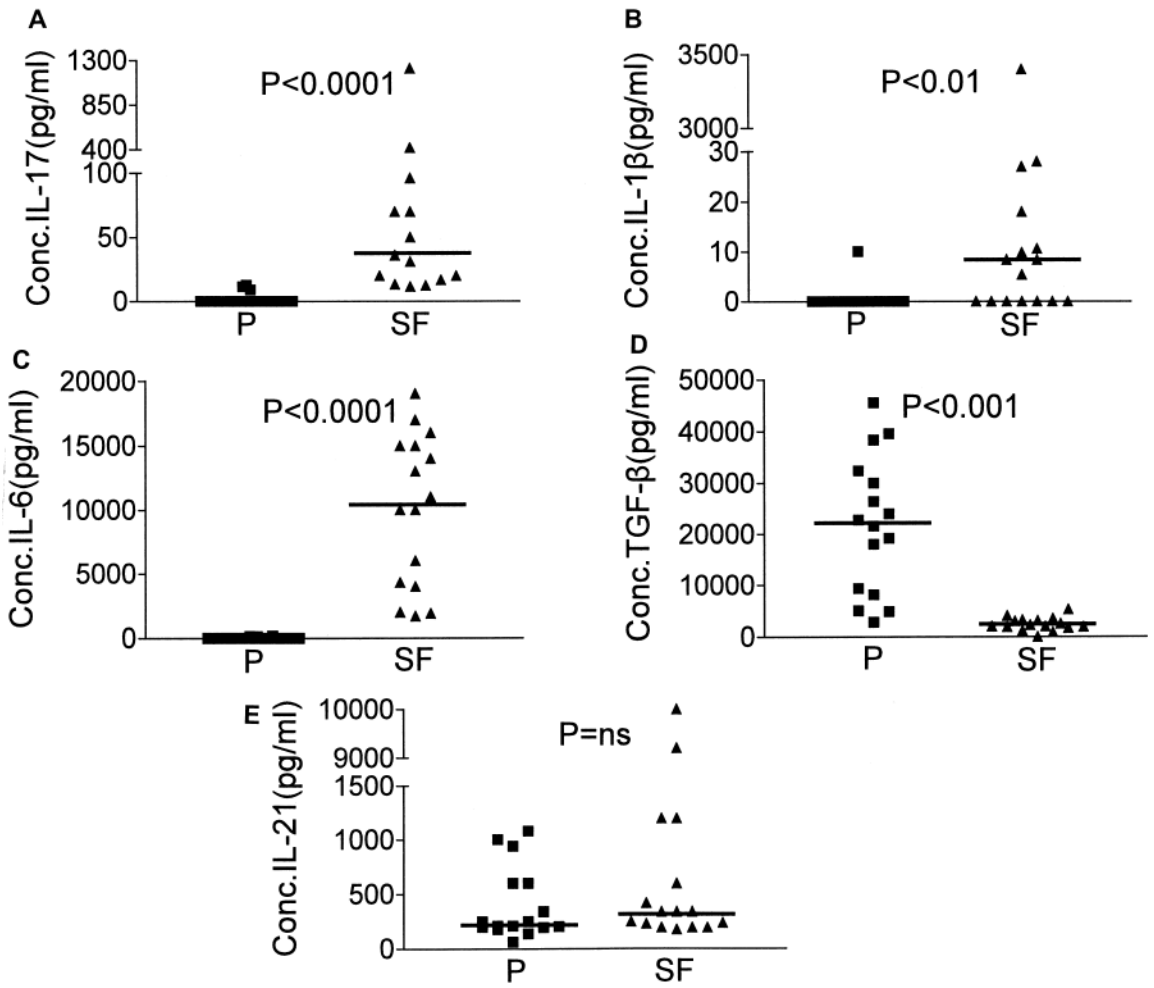

Figure 3. Cytokine concentrations (pg/ml) in simultaneous plasma (P) and synovial fluid (SF) specimens from patients with enthesitis-related arthritis. A. IL-17. B. IL-1ß. C. IL-6. D. TGF-ß. E. IL-21.

effect of MTX on the frequency of Th2 cells or to a more severe disease in such patients.

Recently, Th17 cells have been identified as important proinflammatory mediators in chronic inflammatory diseases. Their role in patients with the ERA type of JIA has not been studied previously. We found a higher frequency of Th17 cells and elevated levels of IL-17 in SF compared to peripheral blood in this group of patients. Nistala, *et al* have shown a similar enrichment of Th17 cells in SF in other subtypes of JIA[7]. A recent study showed that Th17 cells share trafficking receptors with polarized T cells like CCR4, CCR5, and CCR6[21]. Most of these chemokine ligands are present in the SF[16,22] and could mediate trafficking of Th17 to the synovial compartment.

We found high levels of both Th1 and Th17 cell types in the synovial compartment. An inverse relationship has been suggested between these 2 cell types as Th1 cells inhibit Th17 cells, but recent observation of IFN-γ/IL-17-CD4 double-positive cells[7,23,24] gives evidence that Th17 cells could develop by switching lineage from the precommitted cells. This could be due to the abundance of proinflammatory mediators secreted by monocytes/macrophages in the inflamed synovial compartment that mediate differentiation of Th17 cells, either directly from naive T cells or from IFN-γ/IL-17-CD4 double-positive cells.

In human monocyte/macrophage-derived cytokines, IL-6, IL-1ß, and IL-23 are responsible for lineage commitment and maintenance of Th17 cells[23]. Hence our data of higher levels of IL-6 and IL-1ß in SF could suggest their role in Th17 differentiation in SF. Lack of detection of IL-23 in our study is surprising, given that Th17 cells were increased; however, this finding is in agreement with a recent study in RA[25] that showed that only the p19 subunit, and not p40, is elevated in the SF. The assay we used detects only the heterodimeric form of IL-23. Moreover, another study in RA has also shown a lack of IL-23, but increased frequency of Th17, in the SF[26]. Our findings in support of these 2 studies indicate an uncertainty about the role of IL-23 in Th17 polarization in the synovial compartment in ERA.

The role of IL-21 in influencing Th17 differentiation is still

not fully understood, with IL-21–/– cytokine and receptor knockout mice showing normal Th17 development[27]. It is suggested that high IL-6 levels may be sufficient and may compensate the need for IL-21 in mediating autoimmune inflammation[27]. Our observation of markedly increased SF IL-6 levels, but no difference in IL-21 levels, may support this contention. TGF-ß in humans is reported to inhibit Th17 differentiation[23], and low levels of TGF-ß found in the SF may further support Th17 cell differentiation. Thus our findings together suggest that the ERA synovial compartment possesses the ideal environment for Th17 differentiation, and high levels of IL-17 at the site also indicate a Th17-mediated inflammatory response.

Regulatory T cells are believed to reverse immunoinflammation by their ability to inhibit proinflammatory cells. Two studies in JIA patients found higher frequencies of Treg cells in SF than in peripheral blood[11,14]. Among the persistent and extended oligoarticular JIA subtypes, patients with persistent oligoarthritis had higher numbers of Treg cells in the joint, and these Treg were able to suppress CD4+CD25– responder T cells *in vitro*, proving that the clinical course of the disease may be influenced by the presence of functional Treg[14]. Our finding of no difference in the frequency of Treg cells between peripheral blood and SF in ERA is at variance with these studies, and could be related to differences in the disease pathogenesis of ERA. A reciprocal relationship between Th17 and Treg cells has also been reported by Nistila, *et al* in other forms of JIA[7]. Generation of Treg cells can occur either in the thymus or in peripheral lymphoid organs with proper stimulus, and there is clear evidence of TGF-ß having a role in generation of Treg cells from CD4+CD25– T cells in the periphery[28]. Thus we postulate that our observation of no increase in the frequency of Treg cells in ERA SF could be due in part to low levels of TGF-ß in the SF.

Our data suggest that the immunoinflammation in ERA is mediated by Th1 and Th17 cells, supported by the local cytokine milieu. The reduced numbers of antiinflammatory Th2 cells and the lack of increase in numbers of Treg cells could further perpetuate inflammation.

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