IL-27 levels are low in the enthesitis-related arthritis category of juvenile idiopathic arthritis

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Abstract

Objective

Enthesitis-related arthritis (ERA) is associated with the increase in frequency of Th17 cells and synovial fluid IL-17 levels. Recently, IL-15 and IL-18 has been shown to augment and IL-27 to suppress IL-17 production in autoimmune disease, and thus we studied these cytokines in ERA patients.

Methods

Serum samples were collected from 55 patients with ERA, 21 with other categories of JIA and 21 healthy controls. 19 paired synovial fluid samples were also collected from ERA patients. IL-17, IL-23, IL-15, IL-18 and IL-27 levels were measured by ELISA and Th17 frequency was assessed by flow cytometry. IL-23 levels and Th17 frequency was done in subsets of patients.

Results

The median disease duration was 36 months in ERA and 24 months in other JIA subjects. No difference was found in serum levels of IL-17, IL-23, IL-15 and IL-18 between ERA and controls whereas the median IL-27 serum levels were decreased in ERA (107.94 pg/ml (<64-444.40 pg/ml)) as compared to healthy controls (267.28 pg/ml (80.90-492.15 pg/ml); p=0.008). In ERA, synovial fluid levels of IL-17, IL-15 and IL-18 were significantly increased but IL-27 levels were significantly decreased as compared to serum levels. Synovial IL-27 levels correlated negatively with Th17 cell frequency (n=11; r=-0.651, p=0.03).

Conclusion

IL-17 and its supporting cytokines IL-15 and IL-18 are increased whereas its regulatory cytokine IL-27 is decreased in synovial compartment, thus the balance is tilted in favour of IL-17 production. Strategies to increase IL-27 may have therapeutic potential in controlling inflammation at local site.

Key words

inflammation, childhood arthritis, synovitis, pro-inflammatory cytokines, regulatory cytokines

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Introduction

Juvenile idiopathic arthritis (JIA) is the most common cause of childhood chronic arthritis. As per ILAR criteria (1), JIA includes seven mutually exclusive categories and among this enthesitis-related arthritis (ERA) is the most common category of JIA in Asian population including India (2). ERA is characterised by arthritis and enthesitis. The chronic inflammation probably results from a complex interaction between genetic and environmental factors. Sites of inflammation show presence of macrophage, fibroblast, T cell, and synoviocytes. These cells either produce or help in the production of potent pro-inflammatory cytokine interleukin-17 (IL-17). Increased levels of IL-17 have been associated with chronic inflammation in various autoimmune diseases including Ankylosing spondylitis (AS) and JIA (3-5). Major contributors of IL-17 are Th17 cells which are maintained by interleukin-23 (IL-23). In a mice model administration of mini-circles of IL-23 induced enthesitis, major feature of ERA and AS suggesting that IL-23 may be a crucial cytokine in development of these diseases (6). In AS IL-23 receptor positive $CD4^+$ T cells and Th17 cells have positive correlation suggesting the role of IL-23/IL-17 axis (7).

Recently innate immune cells like natural killer (NK) cells, NKT cells, γδ T cells are also found to produce IL-17 (8, 9). In RA, frequency of IL-17 producing NK cells was increased (10). Interleukin-15 (IL-15) has important role in development, expansion and survival of NK cells. In addition it probably increases IL-17 production in RA (11). Synovial membrane of JIA patients has high expression of IL-15 (12), thus IL-15 in JIA may increase IL-17 production. Interleukin-18 (IL-18) synergises with IL-1β and IL-23 to produce IL-17 by innate cells (13). Increased IL-18 levels have been reported in sera of patients with RA and AS (14, 15). IL-18 deficient mice have delayed onset and milder severity of CIA with decreased Th1 response (16). Though serum levels of IL-18 are high in SoJIA and correlate with disease activity (17, 18) no data on serum and synovial fluid IL-18 levels is available in ERA.

Recently, interleukin-27 (IL-27) has been described as a master regulator of inflammation including suppression of IL-17 producing T cells. IL-27 is produced by antigen presenting cells on stimulation with TLR ligands. IL-27 receptor deficient mice have reduced inflammation with decreased IFN-γ production (19). Further IL-27 neutralisation results in suppression of inflammation and bone erosion in CIA and EAE by inhibiting IL-17 and IL-6 production (20, 21). IL-27 is expressed at inflammatory sites in RA, psoriatic arthritis and inflammatory bowel disease (22).

Patients with ERA have high frequency of Th17 in synovial fluid (5) and since cytokines that regulate innate cells can influence them to produce IL-17 and IL-27 can down-regulate IL-17 production we measured the serum and synovial fluid levels of IL-17, IL-23, IL-15, IL-27, IL-18 levels and Th17 frequency in patients with ERA.

Patients and methods

Patients and controls

Patient satisfying the International League of Association of Rheumatology (ILAR) criteria for ERA were included as study subjects (1). In addition children with other categories of JIA were recruited as disease controls. Gender similar young adults were included as healthy controls as it was difficult to get sample from healthy children.

Serum samples were collected and stored at -80° C in aliquots till analysis. Paired synovial fluid was collected from patients with ERA who required intra-articular steroid injection as a part of treatment. No synovial fluid was collected from patients with other categories of JIA. Clinical assessments such as tender joint counts, swollen joint counts were performed by rheumatologist. Erythrocyte sedimentation rate (ESR) was done by Westergren method. Consent was obtained from both patients/parents and healthy controls. Study was approved by the institutional ethics committee.

Measurement of cytokines

Cytokines were measured by sandwich ELISA (eBiosciences, USA) as

Table I. Clinical and demographic details of patients.

Demographic details	ERA (n=55)	Disease control (n=21)
Median age (range: in years)	16 (7-23)	14 (5-26)
Median duration of disease (range: in months)	36 (2-192)	24 (5-192)
Number with active arthritis	48 (87%)	17 (80%)
Number with enthesitis	22 (40%)	None
Number with sacroiliitis	20 (36%)	None
Number with uveitis	3 (5%)	1 (4%)
Inflammatory back pain	13 (23%)	1 (4%)
HLA B27 positive	44 (80%)	1 (4%)

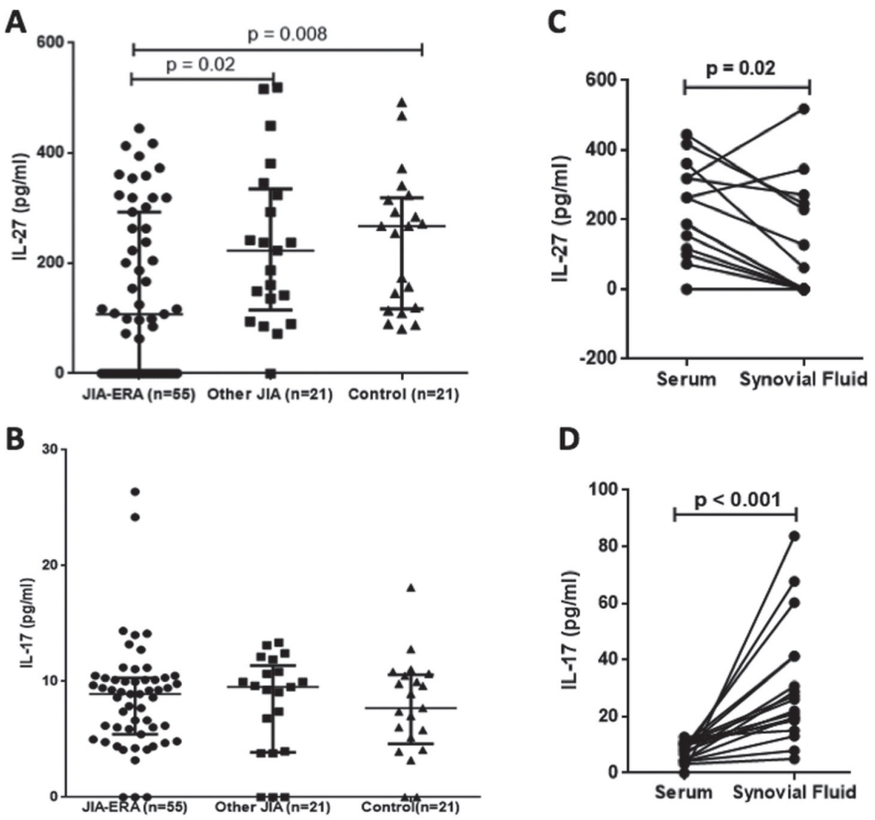

Fig. 1. Scatter plot showing serum cytokine levels of **(A)** IL-27 and **(B)** IL-17 in JIA –ERA (n=55), other JIA (n=21) and Healthy Controls (n=21). Paired values of **(C)** IL-27 and **(D)** IL-17 in serum and synovial fluid of individual patients (n=19).

per manufacturer's instructions. Sensitivity of IL-17, IL-23, IL-15 and IL-27 ELISA kits were 4–500 pg/ml, 15–2000 pg/ml, 8–1000 pg/ml and 64–8000 pg/ml respectively. IL-18 levels were measured by MBL kit (Medical and Biological Laboratories Co Ltd, Japan) with 36.1–257.8 pg/ml sensitivity. Serum and synovial fluid samples were analysed.

Measurement of Th17 cells

500 µl peripheral blood (PB) was cultured in RPMI media supplemented with 10% fetal bovine serum and 1% antibiotic. Cells were stimulated with 50 ng PMA (Sigma, USA) and 1 µg/ml Ionomycin (Sigma, USA). 10 µg/ml brefeldin A (Sigma, USA) was added as secretion inhibitor. PB was cultured in incubator for 6 hrs at 37°C with 5% CO_2. Intracellular staining was done after 6 hours.

Cells were surface stained by anti-CD3 APC and anti-CD4 FITC. After fixation cells were permeabilised using permeabilisation buffer and later stained with intracellular anti-IL-17A PerCP (BD Bioscience, USA) antibodies. $CD4^+$ $IL\text{-}17A^+$ cells were measured in CD3 gate by flow cytometer (Beckman Coulter, USA).

Statistical analysis

Cytokine levels are reported in median (range). Intergroup comparison was done using non-parametric tests. A *p*-value <0.05 was taken as significant. Correlation between cytokines and disease activity was determined by Spearman's correlation coefficient. All analysis was done using SPSS 16 software.

Results

Patients

The study included 55 ERA patients and 21 patients with other forms of JIA as disease controls. Synovial fluid samples were available only from 19 patients with ERA. The median duration of disease was 36 months in ERA and 24 months in other JIA subjects (Table I). Twenty-one age and gender similar healthy control were also included in the study. IL-23 levels were measured only in 39 ERA patients, 18 disease controls and 13 healthy controls. Frequency of Th17 cells were measured in 40 ERA, 12 other JIA, 14 healthy controls and in 11 paired samples.

Cytokine levels in serum and synovial fluid

• *IL-27:* The median IL-27 levels were significantly lower in ERA patients (107.94 pg/ml (<64–444.40 pg/ml)) as compared to disease controls (223.11 pg/ml (<64–518.94 pg/ml); *p*=0.02) and healthy controls (267.28 pg/ml (80.90–492.15 pg/ml); *p*=0.008) (Fig. 1A). IL-27 concentration in synovial fluid (<64 pg/ml (<64–518.94 pg/ml) was further lower as compared to serum levels (170.69 pg/ml (<64–444.4 pg/ml); *p*=0.02) (Fig. 1C).

• *IL-17:* The median IL-17 serum levels in ERA patients were 8.92 pg/ml (<4–26.39 pg/ml), in disease controls were (9.53 pg/ml (<4–13.36 pg/ml) and in healthy control group were 7.71 pg/ml (<4–18.12 pg/ml). No difference was observed in serum levels among three groups (Fig. 1B). However, the median levels of synovial IL-17 were higher (24.07 pg/ml (4.99–83.64pg/ml)) than paired serum IL-17 levels (8.16 pg/ml (<4–12.75 pg/ml); *p*<0.001) (Fig. 1D).

• *IL-23:* IL-23 levels were detectable

in only half of the samples. No difference was observed between ERA and controls. Similarly no difference was observed in serum and synovial IL-23 levels in ERA patients.

• *IL-15:* No difference was found in IL-15 serum levels in patients with ERA (17.87 pg/ml (<8–97.01 pg/ml)), disease controls (17.83 pg/ml (<8–33.6 pg/ml)) and healthy controls (17.47 pg/ml (<8–29.11 pg/ml)) (Fig. 2A). Further IL-15 levels in synovial fluid (27.01 pg/ml (14.92–86.94 pg/ml) were significantly increased as compared to serum (15.13 pg/ml (<4–22.33pg/ml); p=0.004) (Fig 2C).

• *IL-18:* The serum IL-18 levels were also similar in ERA (263.54 pg/ml (88.95-1480.80)) and healthy controls (206.75 pg/ml (42.80-479.05)). Although disease controls had increased IL-18 levels (523 pg/ml (229.30–257.0); p<0.001) as compared to ERA (Fig. 2B). IL-18 levels were also higher in synovial fluid (315.35 pg/ml (165.75–2246.6)) as compared to patient's serum (191.75 pg/ml (88.90–1480.80); p=0.02) (Fig. 2D).

• *Frequency of Th17 cells:* No difference was observed in PB frequency of Th17 cells between ERA patients and healthy controls (Fig. 3A). However the frequency of synovial Th17 cells (1.65%±0.82) was more than PB Th17 cells (0.93%±0.56; p=0.03) in ERA (Fig. 3B).

Correlation of IL-17 and Th17 cells with other cytokines

Serum IL-17 levels had a modest correlation with serum IL-15 levels (r=0.365, p=0.006) and Th17 frequency (r=0.599, p<0.001). Synovial fluid IL-17 levels had a good correlation with synovial fluid IL-18 levels (r=0.692, p=0.013). Synovial Th17 cell frequency negatively correlated with synovial IL-27 concentration (n=11; r=-0.651, p=0.03). Synovial IL-27 and serum IL-27 levels also showed a high positive correlation (r=0.804, p=0.001).

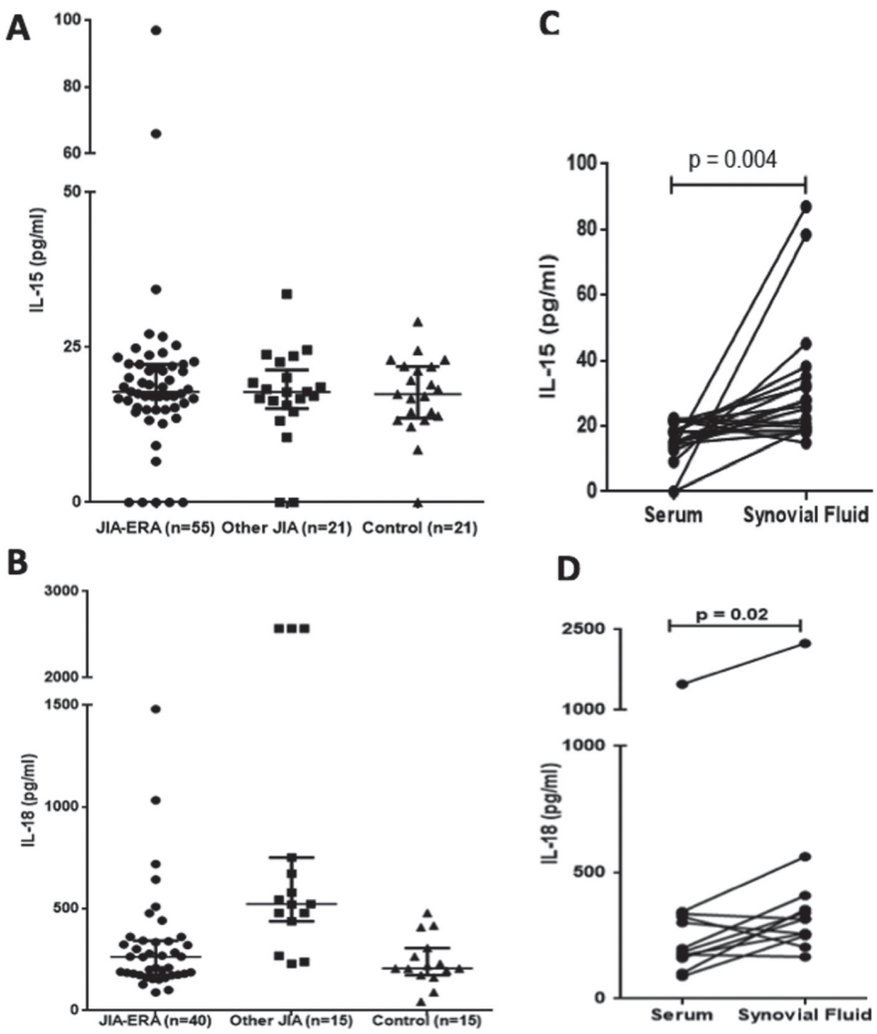

Fig. 2. Scatter plot showing serum cytokine levels of **(A)** IL-15 and **(B)** IL-18 in JIA-ERA (n=55), other JIA (n=21) and healthy controls (n=21). Paired values of **(C)** IL-15 and **(D)** IL-18 in serum and synovial fluid of individual patients (n=19).

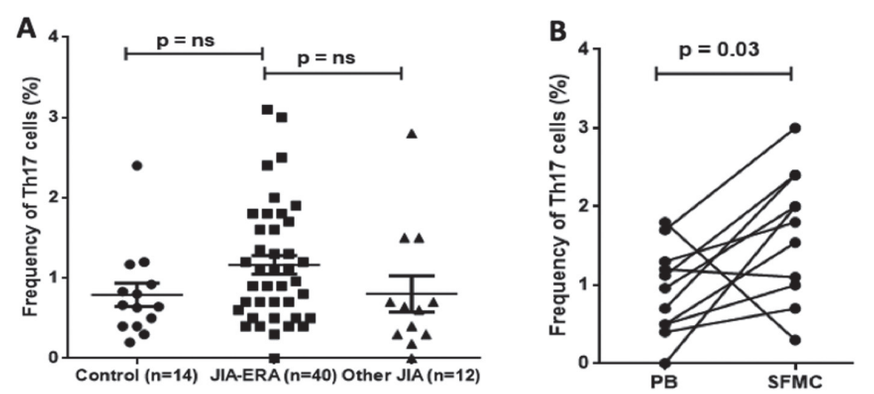

Fig. 3. A. Scatter plots representing the frequency of peripheral blood Th17 cells in healthy controls (n=14), ERA (n=40) and other JIA (n=12). **B.** Paired frequency of Th17 cells in peripheral blood and synovial fluid.

Discussion

Patients with ERA have higher frequency of Th17 cells as well as higher IL-17, IL-15 and IL-18 levels in synovial fluid as compared to blood. In contrast IL-27 levels were lower in synovial fluid as compared to serum levels in patients which were lower than healthy controls. Synovial IL-27 levels had negative correlation with synovial Th17 cell frequency.

A lower serum IL-27 level in ERA as compared to healthy control suggests that in ERA there may be deficient control of inflammation. There is limited data on serum IL-27 levels in human chronic inflammatory diseases. Low levels were also found in patients with systemic lupus erythematosus (SLE) (23, 24). However patients with RA, psoriasis and Crohn's disease had higher expression of IL-27 as compared to healthy control (25-27). This discrepancy could be due to the differential effect of IL-27 on IL-17 production in different inflammatory milieu. IL-27 blocks early Th17 development but fully differentiated Th17 cells are resistant to suppressive effects of IL-27 in presence of IL-23 (28). In ERA we did not find an increase in IL-23 levels thus IL-27 may be able to suppress Th17 cell generation.

Furthermore, the synovial fluid levels of IL-27 were even lower than the corresponding serum levels suggesting that at the local site of inflammation the suppressing ability of IL-27 is further decreased. In CIA and EAE mouse models IL-27 has suppressive effect on Th17 polarisation (20, 21) and IL-27 administration reduces the severity of CIA. Also at synovial site in RA, IL-27 exhibits an anti-inflammatory effect by reducing the IL-6 production and Th17 suppression (29). IL-27 induces suppressor of cytokine signalling 3 (SOCS3) expressions in STAT 1 dependent manner which negatively regulates IL-6 signalling (20) this may leads to the decrease in Th17 polarisation and IL-17 production. Our observation of negative association between synovial fluid IL-27 levels and Th17 cells further supports that low IL-27 is not able to suppress Th17 polarisation. In multiple sclerosis also low plasma IL-27 levels were found and the levels had negative correlation with circulating Th17 cells (30). We however did not find any association between serum levels and Th17 frequency in blood probably as ERA has less systemic inflammation and most immune events occur in the joint and entheses.

Increase in IL-27 production resulting in suppression of Th17 responses may be one of the mechanism by which corticosteroids work in chronic inflammatory diseases as was seen after the administration of cyclosporine and steroids (31). Other mechanisms contributing to anti-inflammatory effect include IL-27 induced IL-10 production by regulatory T cell (28) and increased expression of CD39 (32) which acts via increasing extracellular adenosine. Recently a rise in urinary IL-27 level was seen with immunosuppressive treatment for lupus nephritis (33).

Our data of increased IL-17 levels in synovial fluid is similar to previous reports in JIA (5). Increased frequency of synovial Th17 cells suggests their contribution in sustained inflammation. IL-23 levels were not different between patients with ERA and controls. Recently in AS also no difference was found in circulating IL-23 and IL-17a levels in patients and healthy controls (34). Furthermore, a study in RA found abundant expression of IL-23p19 subunit but heterodimeric IL-23 was not expressed in TLR stimulated RA synovial fibroblast suggesting the differential expression of IL-23 and its subunits (35). We have only looked for the heterodimeric IL-23, thus it would be interesting to measure the IL-23 p19 levels in ERA.

We also found higher levels of IL-15 in synovial fluid of ERA patients as compared to serum and serum IL-15 levels correlated with IL-17 levels. IL-15 is known to stimulate IL-17 production in RA (11). Thus IL-15 may have synergetic effect on IL-17 production or both may be induced by the same stimuli and together they contribute to inflammation. IL-15 also plays a role in development and differentiation of NK cell, NKT cells and these cells are also known to produce IL-17 in pathological condition (8).

Higher synovial IL-18 levels as compared to serum IL-18 in ERA suggest that they may be produced locally and play a role in inflammation. IL-18 acts synergistically with IL-1 and IL-23 to maintain IL-17 producing cells (13). In addition IL-18 also increases TNF alpha and IFNγ production thus contributing to inflammation. IL-18 serum levels in SoJIA and synovial fluid levels in RA correlate with disease activity (14, 18), however, in AS no correlation was found with disease activity (15).

Thus our data suggests that low levels of IL-27 may not be able to control inflammation mediated by IL-17 as reflected by elevated IL-17 levels and frequency of Th17 cells. Further elevated IL-15 and IL-18 may increase IL-17 production by innate immune cells thus contributing to inflammation. Therapies targeted at IL-15, IL-18 and IL-17 have shown some promise in other immune-inflammatory diseases (36-39) and may have a role in ERA. Another possible strategy is to increase IL-27 levels by drugs like corticosteroids or biologics.

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