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Research Article

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Research Article

IL-36γ in enthesitis-related juvenile idiopathic arthritis and its association with disease activity

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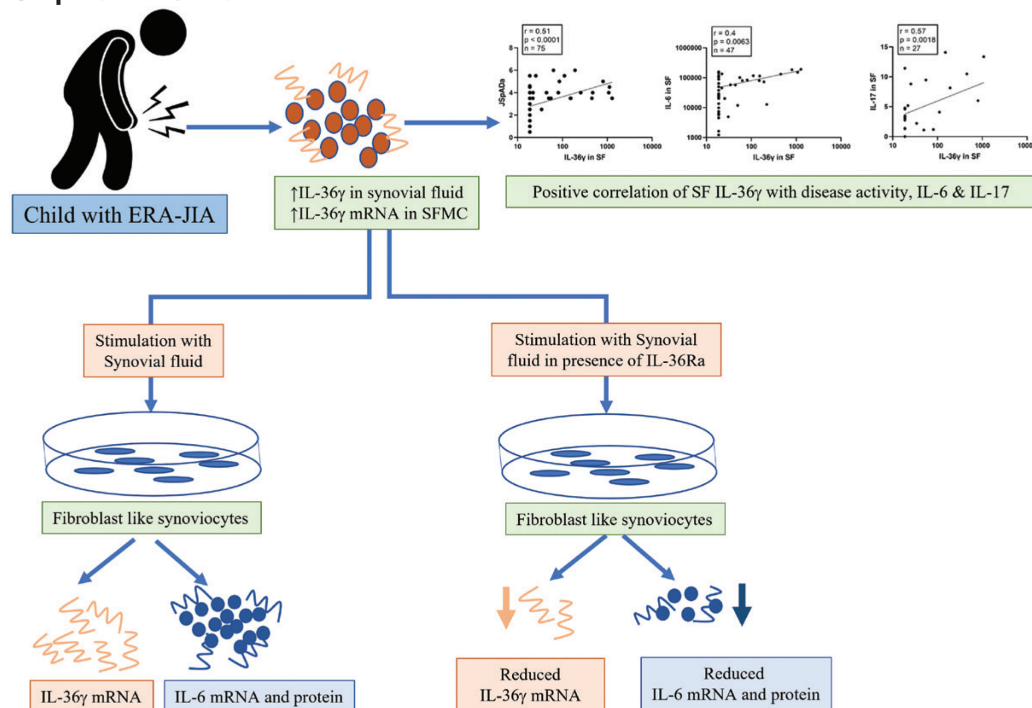
Abstract

IL-36 has been implicated in the pathogenesis of spondyloarthropathies (SpA) like psoriasis and inflammatory bowel disease. Enthesitis-related arthritis (ERA) category of juvenile idiopathic arthritis is a form of juvenile SpA, however, no data is available on the role of IL-36 in this disease. IL-36α, β, γ and IL-36R mRNA expression in blood and synovial fluid mononuclear cells and IL-36α, γ, IL-36Ra, IL-6, and IL-17 levels were measured in serum and synovial fluid (SF). IL-36γ production by fibroblast-like synoviocytes (FLS) upon stimulation with pro-inflammatory cytokines and its effect on FLS were also studied. mRNA levels of IL-36α, IL-36γ, and IL-36R were increased in PBMCs of ERA patients as compared to healthy controls however only IL-36γ was measurable in the serum of one-third of patients. In SFMCs, all four mRNA were detectable but were lower than RA patients. SF IL-36γ levels correlated with disease activity score ($r = 0.51$, $P < 0.0001$), SF IL-6 ($r = 0.4$, $P = 0.0063$) and IL-17 levels ($r = 0.57$, $P = 0.0018$). Pro-inflammatory cytokines increased the expression of IL-36γ and IL-6 in FLS cultures. SFs from five ERA patients also increased expressions of IL-36γ and IL-6 in FLS which could be blocked by using IL-36Ra. This suggests that pro-inflammatory cytokines aid in the upregulation of IL-36γ which in turn may upregulate the expression of IL-6. This might lead to a positive feedback loop of inflammation in ERA. Association of SF levels of IL-36γ with disease activity further supports this possibility. IL-36Ra based therapy may have a role in ERA.

Graphical Abstract



Keywords: IL-36, cytokine, ERA-JIA, inflammation, spondyloarthropathy

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Abbreviations: ELISA: enzyme-linked immunosorbent assay; ERA-JIA: enthesitis-related arthritis-juvenile idiopathic arthritis; FLS: fibroblast-like synoviocytes; IBD: inflammatory bowel disease; IL: interleukin; ILAR: international league of associations for rheumatology; JSpaDA: juvenile spondyloarthropathy disease activity score; PBMC: peripheral blood mononuclear cells; PBS: phosphate-buffered saline; PsA: psoriatic arthritis; RA: rheumatoid arthritis; RT-qPCR: real-time quantitative polymerase chain reaction; SF: synovial fluid; SFMC: synovial fluid mononuclear cells; SpA: spondyloarthropathy.

Introduction

Juvenile idiopathic arthritis (JIA) is the most common form of childhood rheumatic disease and encompasses a heterogeneous group of arthritic diseases. Among its different subtypes, enthesitis-related arthritis (JIA-ERA) is one of the most common forms of JIA in southeast Asia including India and constitutes 30–35% of patients, in contrast to only 5–10% in Western countries. It resembles adult spondyloarthropathies (SpA) with which it shares male preponderance, presence of enthesitis, lower limb arthritis, sacroiliitis, and HLA B27 association where HLA B27 prevalence varies from 60% to 90% in different forms of SpA [1, 2].

Although the pathogenesis of JIA-ERA is unclear, recent data shows evidence of gut dysbiosis suggesting a role in the interaction between HLA B27 and the gut microbiome. Gut microbes, by activating TLR mediated pathway and HLA B27 induce an unfolded protein response (UPR) that results in the activation of monocyte-macrophage lineage cells. This leads to the production of pro-inflammatory cytokines including IL-23 by these cells. IL-23 in turn acts on IL-23R bearing T cells, innate lymphoid cells, and NK cells to cause the production of IL-17 and IL-22, the two key cytokines involved in inflammation in joints and enthesitis. In addition, other proinflammatory cytokines like IL-6, TNF-alpha, IL-8, and IL-15 are also produced which enhance the inflammation [3–10].

Recently, the IL-1 family member IL-36 (having isoforms α, β, and γ) and its receptor antagonist IL-36Ra have also been implicated in the pathogenesis of psoriasis and inflammatory bowel disease (IBD) both of which can precipitate SpA. Psoriatic skin lesions show upregulation of all three isoforms of IL-36, and keratinocytes derived from psoriatic lesions produce IL-36 in response to TNF-α, IL-17, and IL-22. Mice over-expressing IL-36α develop psoriasis-like skin lesions and deletion of the IL-36Ra gene in them increases disease severity [11, 12]. Synovial lining in patients with psoriatic arthritis (PsA) as well as rheumatoid arthritis (RA) also shows higher expression of IL-36 compared to osteoarthritis [13–17]. The synovial plasma cells were identified as the primary source of IL-36, which in turn induced the production of inflammatory cytokines by fibroblast-like synoviocytes (FLS) [11]. In animal models of psoriasis, blockage of IL-36R results in disease amelioration accompanied by a reduction in IL-17, leukocyte infiltration and keratinocyte activation [18–20]. Most importantly, IL-17 stimulates keratinocytes to produce IL-36 which in turn induces IL-17 production leading to a positive feedback loop in psoriasis [21], possibly resulting in the dysregulated state of the immune response. However, in RA, IL-36 expression does not seem to correlate with the IL-17 signal [11, 17].

As mentioned above, JIA-ERA, IBD, and psoriasis belong to the broad group of SpA, but not RA since the synovitis in RA is different from that in SpA. We, therefore, hypothesized that, like PsA and IBD, IL-36 may also be associated with synovitis in JIA-ERA and contribute to the dysregulated inflammatory response of the synovium, accompanied by the production of other pro-inflammatory cytokines.

Materials and methods

Patients

Fifteen ERA patients fulfilling the JIA-International League of Associations for Rheumatology (ILAR) classification criteria [22] were included in the study. None of the patients had psoriasis or IBD. Clinical details were collected, and disease activity was assessed using Juvenile Spondyloarthropathy Disease Activity Score (JSpaDA) by the treating rheumatologist at the time of sample collection. As controls, blood from 10 young adult healthy males and blood and synovial fluid (SF) from five patients with RA undergoing joint aspiration were also obtained. Approval from the Institutional Ethics Committee was obtained prior to commencement of the study (IEC approval code: 2018-10-EMP-102) and all patients/guardians provided written informed consent. We have also used previously stored synovial fluid and serum samples from 69 patients with JIA-ERA, 15 patients with RA, and 14 healthy subjects. A total of 84 patients were included in the study. All clinical details of patients are listed in Table 1.

Isolation of PBMCs and SFMCs

Peripheral blood mononuclear cells (PBMC) and synovial fluid mononuclear cells (SFMC) were isolated by density gradient centrifugation. Briefly, diluted heparinized blood or diluted pelleted synovial fluid cells were layered on Histopaque 1077 (Sigma–Aldrich, St Louis, MO, USA) and centrifuged (1800 rpm, 30 min). The interface containing mononuclear cells was collected and washed (×3) with PBS.

RNA isolation, cDNA synthesis, and gene expression studies using RT-qPCR

RNA was isolated from PBMCs and SFMCs by the Trizol-Chloroform extraction method and cDNA was synthesized utilizing the High-Capacity cDNA synthesis kit (Invitrogen, USA). Real-time PCR was performed using the SYBR Green Master mix (Invitrogen, USA). Primer sets used for RT-qPCR are listed in Supplementary Table S1. All RT-qPCR analyses were performed in the Roche Light Cycler 480 instrument (Roche, USA). Melting curve analyses were performed for every experiment to rule out non-specific mRNA amplification. Fold change calculation was done using the ΔΔCt method. Results were denoted either by fold difference (compared to healthy control) or difference in Ct values (ΔCt) of the gene of interest and housekeeping genes.

Fibroblast like synoviocyte (FLS) culture

SF was collected from a JIA-ERA patient in a heparinized tube and centrifuged (1800 rpm, 10 min). The cell pellet was re-suspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and plated in a tissue culture flask. Cells were incubated at 37 °C in 5% CO_2 for 48 h. The medium was aspirated and the cell monolayer was washed with PBS to remove any non-adherent cells prior to the addition of fresh medium.

FLS cultures from synovial biopsies were done as previously described [23]. Briefly, perioperatively obtained synovial tissues from patients undergoing joint replacement (n = 4)

Table 1: Clinical characteristics of patients with JIA-ERA.

Variables	Patients from whom PBMCs or SFMCs were obtained	Patients from whom serum were obtained	Patients from whom synovial fluid were obtained	Patients from whom paired serum and SF were obtained	Total patients
Number of patients	15 (13M + 2F)	62 (58M + 4F)	75 (68M + 7F)	48 (45M + 3F)	84 (77M + 7F)
Age (years)	15(4.5)	17(3.5)	16 (4)	16 (3.5)	17 (5)
Duration of disease (months)	54 (71.75)	60 (69)	60 (72)	60 (69)	60 (72)
ESR (mm)	68(66)	64 (55.5)	56 (49.5)	62 (56)	57 (55)
JSpaDA score	2.5 (1.5)	3.5 (1.8)	3.5 (2)	3.5 (1.8)	3.5 (2)
DMARD usage	12 (8 on MTX and 4 on SSZ)	16 (10 on MTX and 6 on SSZ)	20 (13 on MTX and 7 on SSZ)	17 (10 on MTX and 7 on SSZ)	21 (14 on MTX and 7 on SSZ)
Patients on corticosteroids	1	2	2	2	3

All values are median and IQR.

were finely minced and treated with collagenase A (37 °C, 90 min). After removing collagenase, cells were washed (×3) with PBS and allowed to adhere (48 h) in cell culture flasks. Non-adherent cells were removed with PBS washes and a fresh complete medium (DMEM + 5% FBS) was added. The medium was replaced every 3–4 days and routinely split after reaching confluency. All experiments were performed when FLS were between the 4th and 6th passages.

For gene expression studies, FLS were seeded in 6-well culture plates (2×10^5 cells/well) and allowed to adhere for 24 h. Cells were initially starved for serum (2 h) and then stimulated for 3, 6 or 24 h with complete medium containing IL-36γ (1 µg/ml), IL-6 + IL-6R (100 ng/ml + 100 ng/ml), IL-17 (50 ng/ml), IL-6 + IL-6R + IL-17, TNF-α (50 ng/ml) or medium alone. All recombinant protein concentrations were used as previously reported [24].

To study the effect of SF from JIA-ERA patients on FLS, cells were stimulated with SF from five JIA-ERA patients having detectable levels of IL-36γ (10% SF in complete medium), in the presence or in the absence of IL-36Ra (1 µg/ml). All simulations were done in duplicates. Post-stimulation, cells were harvested and stored in trizol for RNA extraction. RT-qPCR was performed with the cDNA reverse transcribed from isolated RNA samples.

For ELISA, FLS were seeded in 96-well plates (10^4 cells/well) and stimulated (8 h, in triplicate) with the stimulants as previously described. Later, stimulants were removed, and cells were washed (×3) with PBS. Cells were replenished with a fresh complete medium and allowed to rest overnight. Supernatants were stored and cell viability was assessed using an MTT assay.

ELISA

Clotted blood and SF were centrifuged (1800 rpm, 10 min) and serum and cell-free SF were stored at −80 °C in aliquots. ELISAs for IL-36α, IL-36γ, IL-36 Ra, IL-6, and IL-17A were performed using ELISA kits (R&D Systems, USA or BD Biosciences, USA). The minimum detection limit for IL-17 was 15.6 pg/ml; IL-6 was 9.38 pg/ml; IL-36γ was 18.75 pg/ml; IL-36α was 12.5 pg/ml; IL-36Ra was 93.8 pg/ml. All ELISAs were performed by following the manufacturer's protocols.

Statistical analyses

The data were analyzed using Graph Pad Prism 8.0 Software (GraphPad Software Inc. San Diego, CA, USA). All clinical parameters and cytokine levels (protein and mRNA) are expressed as the median and interquartile range (IQR). Non-parametric Mann–Whitney *U* test and Wilcoxon signed-rank test were used for intergroup comparisons. Proportion analysis was done using the Chi-square test with Yates correction if needed. Correlation analysis was performed using Spearman's rank correlation test. A P value of <0.05 was considered statistically significant.

Results

Clinical characteristics of JIA-ERA patients

For mRNA expression studies, cells from the 15 enrolled JIA-ERA patients and controls (RA patients and healthy subjects) were used. In addition, for SF and serum studies, previously stored samples were also included from 69 patients. A total of 84 JIA-ERA, 20 RA, and 24 healthy subject samples were used for ELISA, out of which 48 were paired samples (both serum and SF). Among the 84 JIA-ERA patients, 77 were boys. Their median age was 17 years and disease duration was 60 months. All had active disease with a median ESR of 57 mm and JSpaDA of 3.5, The median age of healthy subjects was 19 years. Out of 84 JIA-ERA patients, 21 were on disease-modifying anti-rheumatic drugs (DMARDs), 3 patients were on corticosteroids and the rest were on treatment with non-steroidal anti-inflammatory drugs (NSAIDs). (Table 1)

Patient mononuclear cells showed increased expression of IL-36α, IL-36γ, and IL-36R

PBMCs of JIA-ERA patients (n = 15) had higher levels of IL-36α, IL-36γ, and IL-36R mRNA as compared to healthy controls (n = 10) and no difference was observed in the expression levels of IL-36β (Fig. 1A). Though mRNA expression of all four genes was observed in SFMCs of JIA-ERA patients, it was lower than that seen in RA (Fig. 1B).

Serum and SF levels of IL-36γ were significantly higher in JIA-ERA compared to healthy subjects

Serum IL-36γ was detected in 21 of 61 JIA-ERA patients and only 1 of 24 healthy subjects (P < 0.005). The median value was higher in patients as compared to healthy control (P < 0.01; Fig. 2A). In SF, it was present in 24 of 75 JIA-ERA

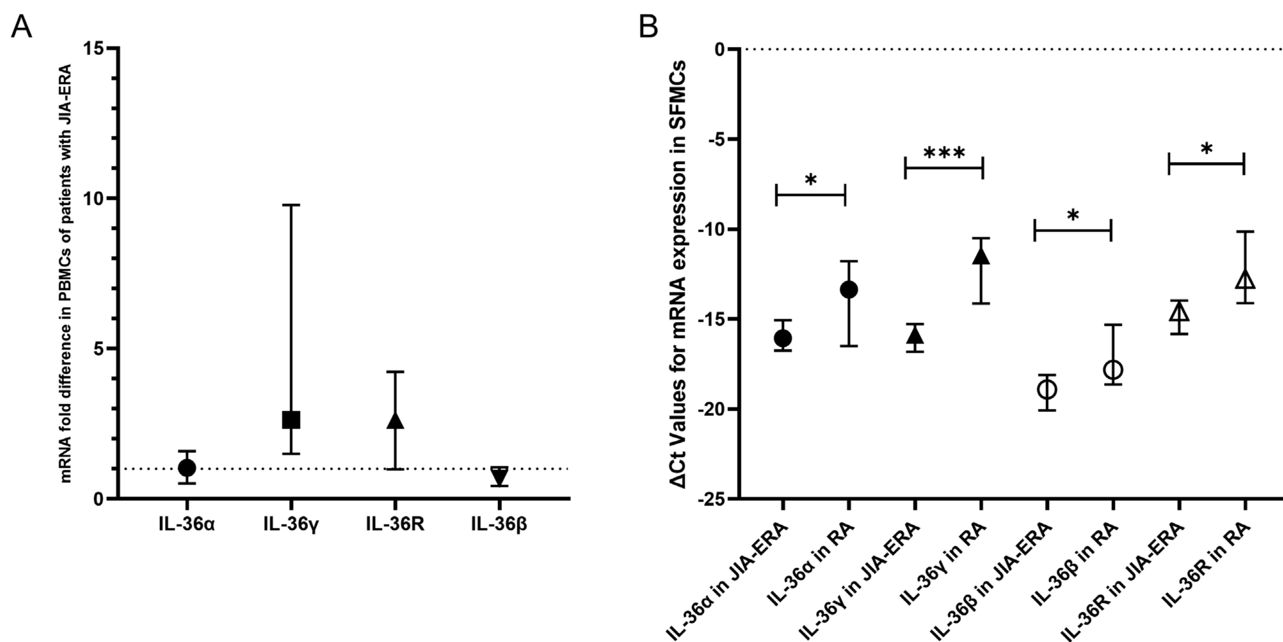

Figure 1: mRNA expression of IL-36 genes in PBMCs and SFMCs of patients with JIA-ERA. (A) Fold difference in mRNA expression of IL-36 cytokine genes in PBMCs of JIA-ERA patients compared to healthy controls. (B) ΔCT values depicting differences in mRNA expression of IL-36 genes in SFMCs of JIA-ERA patients compared to RA patients. All data are represented as median (IQR). Significance was determined by s-parametric Mann–Whitney *U* test (*$P \le 0.05$, ***$P \le 0.001$). *n*(JIA-ERA) = 15, *n*(HC) = 10, *n*(RA) = 5.

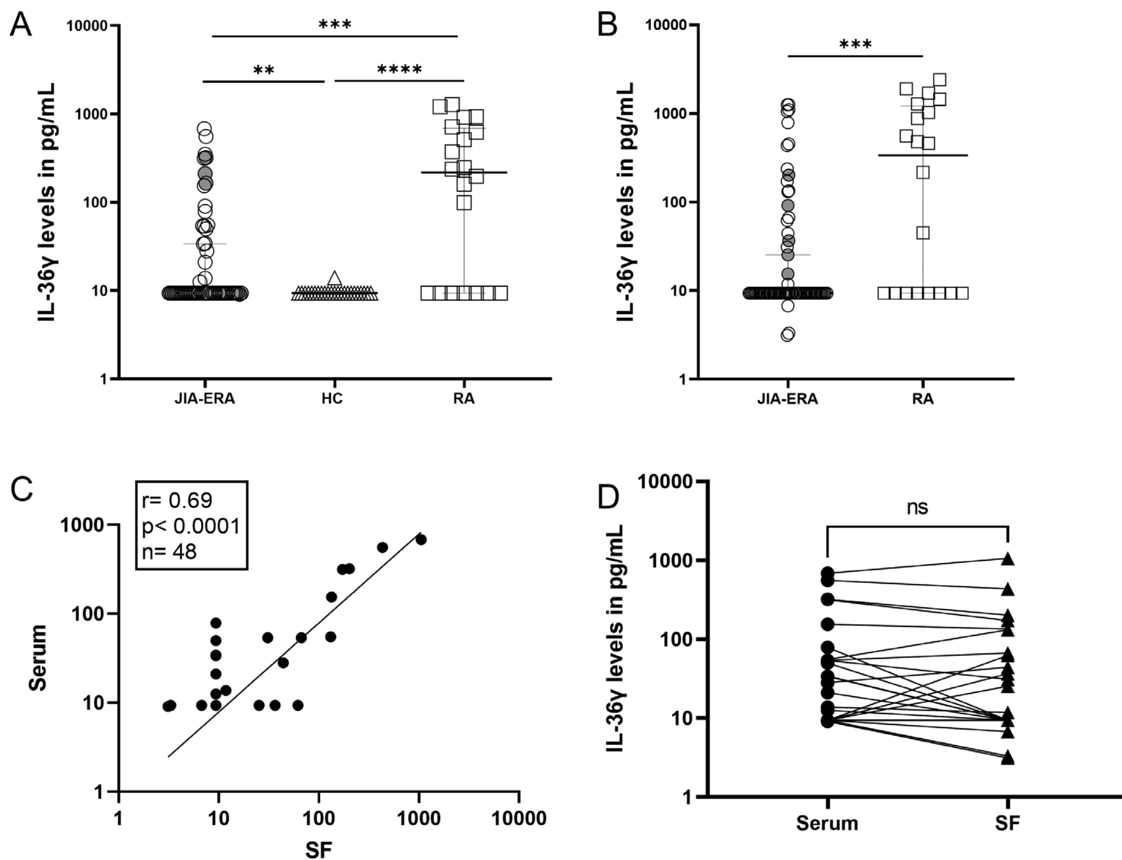

Figure 2: IL-36γ levels in serum and synovial fluids of patients with JIA-ERA. (A) IL-36γ levels in serum. (B) IL-36γ levels in synovial fluid. (C) Correlation of IL-36γ levels in serum and synovial fluid. (D) Comparison of serum and synovial fluid levels of IL-36γ. All data are represented as median (IQR). Significance was determined by non-parametric Mann–Whitney *U* test (*$P \le 0.05$, ***$P \le 0.001$ and ****$P \le 0.0001$). *n*(JI-ERA) = 84, *n*(RA) = 20, *n*(HC) = 24.

samples (Fig. 2B). However, IL-36γ levels in the serum as well as SF of patients with JIA-ERA were lower than in patients with RA patients. IL-36α was detected in the serum and SF of only one JIA-ERA patient and none of the healthy sera. IL-36Ra was also not found in any sample (data not shown). In paired samples, serum and SF levels of IL-36γ showed a positive correlation ($r = 0.69$, $P < 0.0001$, Fig. 2C and D), but were not significantly different from each other.

Correlation of SF IL-36γ levels with disease activity and other cytokines

Patients with JIA-ERA had significantly higher serum and SF levels of IL-6 and IL-17 than the healthy subjects (Fig. 3A and C). In patients, SF levels of both cytokines were significantly higher than the serum level (Fig. 3B and D). Serum IL-36γ positively correlated with disease activity score JSpaDa in DMARD naïve patients ($r = 0.44$, $P = 0.0017$) (Supplementary Fig. 1). SF IL-36 γ levels correlated positively with JSpADA ($r = 0.51$, $P < 0.0001$) IL-6 and IL-17 levels ($r = 0.40$, $P = 0.0063$ and $r = 0.57$, $P = 0.0018$, respectively; Fig. 3E–G).

Effect of proinflammatory cytokines on FLS

Stimulation of FLS with pro-inflammatory cytokines increased the mRNA expression of both IL-6 and IL-36γ and protein expression of IL-6 (Fig. 4A–C). TNF-α was found to be the most potent inducer of IL-36γ mRNA expression from FLS. (Fig. 4B). IL-6 mRNA expression induced by pro-inflammatory cytokines peaked at 6 h and IL-36γ mRNA expression was the highest at 3 h, reducing thereafter (Supplementary Fig. S2A and B).

Effect of SF from patients with JIA-ERA on FLS and impact of IL-36Ra treatment

Stimulation of FLS with SF from five JIA-ERA patients (who had detectable levels of IL-36γ) enhanced the mRNA expression of both IL-6 and IL-36γ as compared to controls. However, in the presence of IL-36Ra, the SF-induced expression of both genes was significantly reduced ($P < 0.0001$; Fig. 5A and B). IL-6 protein expression was also induced in the presence of SF and reduced significantly when stimulated in presence of IL-36Ra ($P < 0.0001$; Fig. 5C). This further strengthened the notion that IL-36γ can induce the expression of pro-inflammatory cytokines like IL-6 as well as of IL-36γ itself.

Discussion

JIA-ERA patients had higher expression of IL-36γ as well as higher serum levels compared to healthy controls. Further SF IL-36γ correlated with SF IL-17 and IL-6 levels as well as with disease activity.

Our finding of enhanced IL-36γ expression in PBMC and its higher serum levels in JIA-ERA is similar to that reported for other rheumatic diseases including PsA, RA [25]. IL-36γ was also found significantly increased in SLE patients with arthritis compared to those without arthritis [26]. In psoriasis,

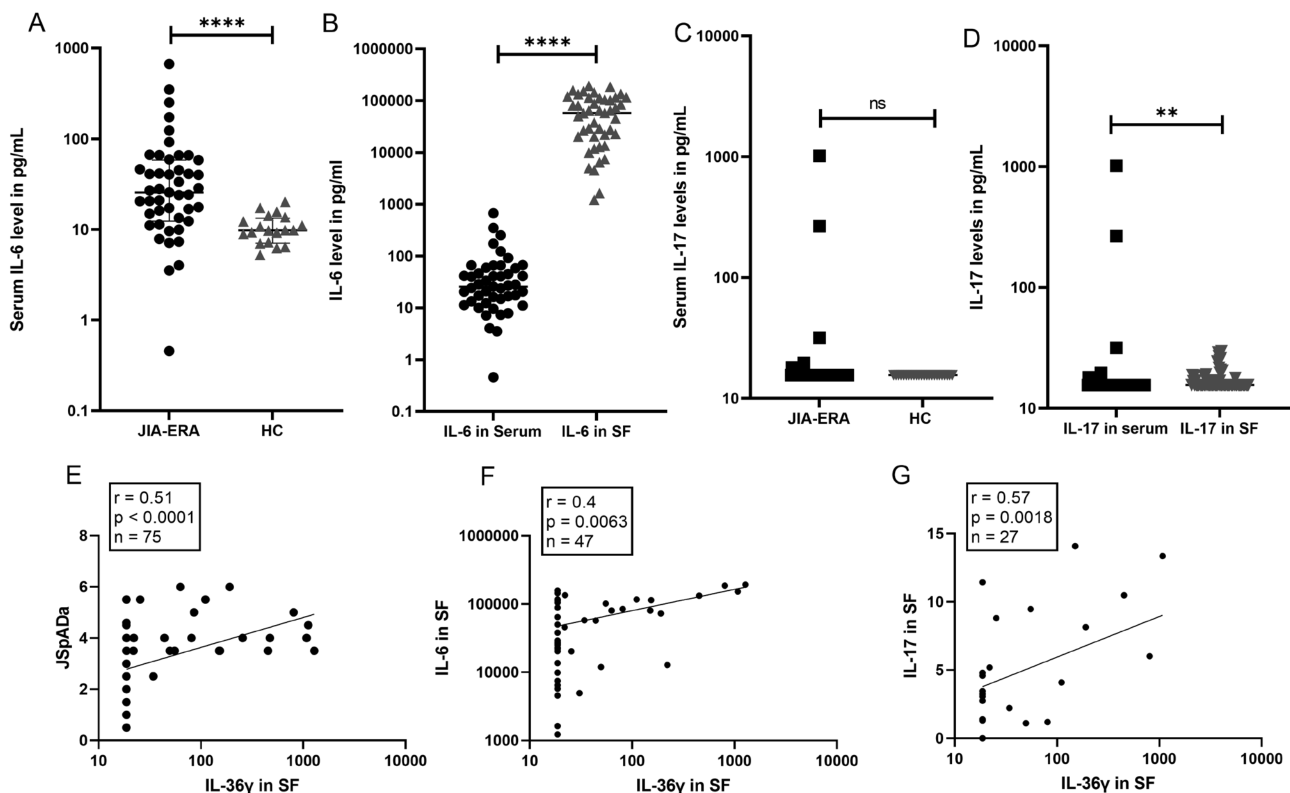

Figure 3: Correlation of IL-36γ with disease activity and pro-inflammatory cytokine levels. (A) Serum IL-6 levels in patients with JIA-ERA compared to healthy control (B) Comparison of serum and synovial fluid levels of IL-6 in patients with JIA-ERA (C) serum IL-17 levels in patients with JIA-ERA compared to healthy control (C) Comparison of serum and synovial fluid levels of IL-17 in patients with JIA-ERA (E) Correlation of synovial IL-36γ levels with disease activity score JSpaDA. (F) Correlation of synovial IL-36γ levels with synovial IL-6 levels. (G) Correlation of synovial IL-36γ levels with synovial IL-17 levels. All data are represented as median (IQR). Significance was determined by non-parametric Mann–Whitney *U* test (*$P \leq 0.05$, ***$P \leq 0.001$ and ****$P \leq 0.0001$). *n*(JI-ERA) = 84, *n*(HC) = 24.

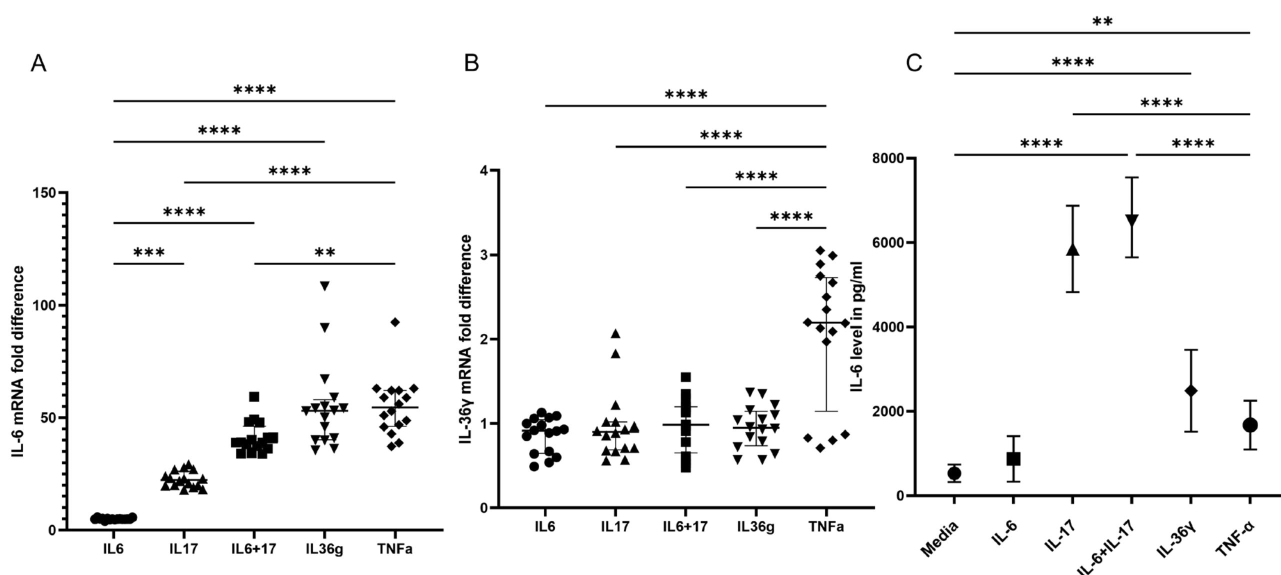

Figure 4: Expression of IL-36γ and IL-6 in FLS upon stimulation with pro-inflammatory cytokines. (A) Difference in mRNA expression of IL-6 in FLS upon stimulation with pro-inflammatory cytokines compared to unstimulated controls at 6 h. (B) Difference in mRNA expression of IL-36γ in FLS upon stimulation with pro-inflammatory cytokines compared to unstimulated controls at 3h. (C) IL-6 levels in FLS cell supernatant post-stimulation with pro-inflammatory cytokines. All data are represented as median (IQR). Significance was determined by one way analysis of variance (ANOVA) (** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$, ns = non-significant); n(FLS) = 10.

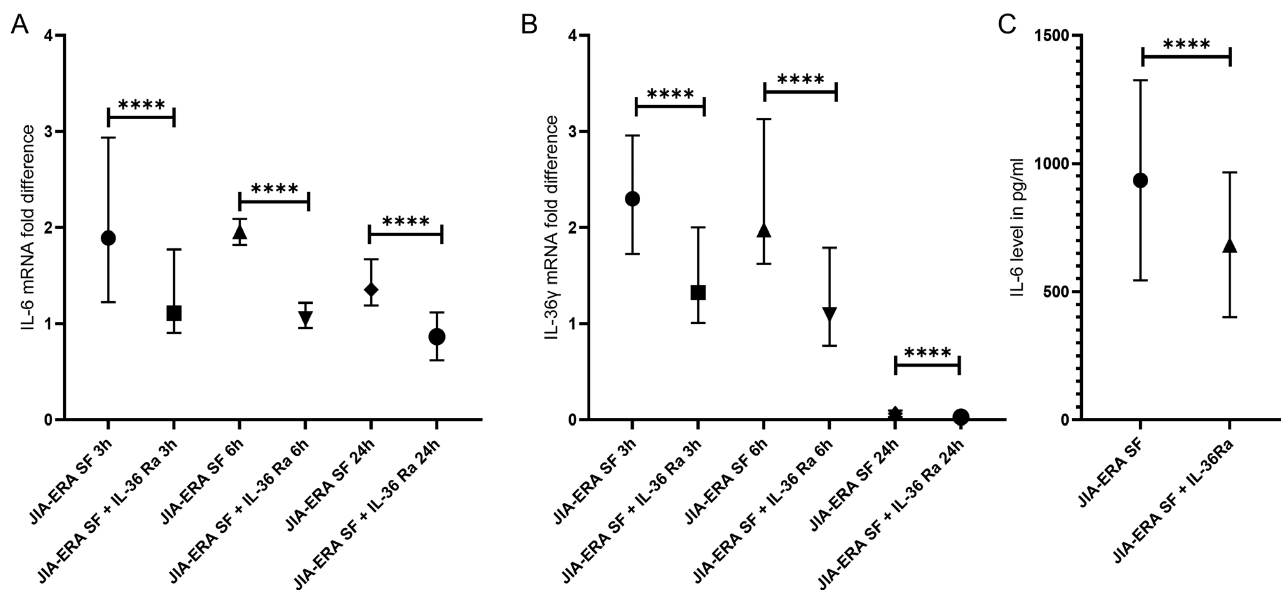

Figure 5: Expression of IL-36γ and IL-6 in FLS upon stimulation with synovial fluids of patients with JIA-ERA in the presence or in the absence of IL-36Ra. (A) Difference in mRNA expression of IL-6 in FLS upon stimulation with synovial fluids of patients with JIA-ERA in the presence or in the absence of IL-36Ra. (B) Difference in mRNA expression of IL-36γ in FLS upon stimulation with synovial fluids of patients with JIA-ERA in the presence or absence of IL-36Ra. (C) IL-6 levels in FLS cell supernatant post-stimulation with synovial fluids of patients with JIA-ERA in the presence or in the absence of IL-36Ra. All data are represented as median (IQR). Significance was determined by non-parametric Mann–Whitney U test (**** $P \leq 0.0001$). n(FLS) = 10.

rheumatoid arthritis, and SLE also, the expression of IL-36 cytokines correlated significantly with disease activity [11, 15, 26, 27, 28]. This suggests that IL-36γ may have a role in the pathogenesis of synovitis.

High levels of IL-36γ in SF and its correlation with disease activity and SF IL-6 and IL-17 levels suggest its importance in the local milieu. Skin biopsies of psoriasis patients, intestinal tissue of IBD patients, synovial tissue of patients with psoriatic arthritis, and RA and urine of lupus nephritis patients have also shown localized expression of IL-36 further supporting its role in local inflammation [13, 14, 17, 25, 28, 29].

A positive correlation of SF IL-36γ with IL-6 and IL-17 levels could either suggest that all three proinflammatory cytokines are produced in response to the same stimuli or alternatively, IL-36 may enhance the production of these

cytokines. In this context, we did observe increased expression of mRNA as well as IL-6 production by FLS upon exposure to IL-36γ. In a previous study, IL36α was shown to induce IL-6 and IL-8 production in synovial fibroblasts [25]. IL-36 cytokines were also found to induce the production of IL-6 in human colonic subepithelial myofibroblasts [17, 30, 31] further supporting our finding of IL-36γ as an inducer of pro-inflammatory cytokines.

The *in vitro* effects of SF from JIA-ERA on FLS further supported role of IL-36γ. It suggested that IL-36γ could induce the production of IL-6 and IL-36γ and may drive inflammation in JIA-ERA. Besides IL-17, IL-6 is also an important cytokine associated with synovial inflammation of JIA-ERA [5]. Similar to our observation of inhibition of IL-6 production by IL-36Ra in FLS stimulated by patients SF, the previous study has shown a reduction in IL-8, IL-17, and IL-22 production in PBMCs induced by IL-36γ [32]. Boutet et al. have shown that IL-36γ-induced production of IL-8 by human PBMCs was also reduced by IL-36Ra [25]. However, to the best of our knowledge, no previous studies have reported reduced IL-6 or IL-36 production in the presence of IL-36Ra.

One of the limitations of our study is the inclusion of patients with significant disease duration. It would have been ideal to study patients in the early phase of the disease, as the inclusion of patients with longer disease duration may result in a bias toward patients with persistent disease. Thus, our findings need to be validated in a cohort of early ERA patients.

To conclude, our results suggest that, among the IL-36 family of pro-inflammatory cytokines, IL-36γ may have a role in the synovial inflammation seen in a subset of JIA-ERA patients. Since TNF-α increases IL-36, anti-TNF agents currently licensed for use in ERA may be beneficial in blocking this axis. However, on chronic use patients develop secondary resistance to anti-TNF therapy and alternative therapies are needed. And as Spesolimab and Imsidolimab, the IL-36 receptor antibodies are currently undergoing clinical trials and showing promise in the treatment of general pustular psoriasis and palmoplantar pustulosis [16, 18, 33, 34], these may also have some role in a subset of patients with JIA-ERA with high IL-36γ levels.

Supplementary data

Supplementary data is available at *Clinical and Experimental Immunology* online.

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Conflicts of interest

The authors declare that there is no conflict of interest.

Author contributions

Study conception and design: Sanjukta Majumder, Amita Aggarwal; Data collection: Sanjukta Majumder, Shivika Guleria; Analysis and interpretation of results: Sanjukta Majumder, Amita Aggarwal; Draft manuscript preparation: Sanjukta Majumder, Amita Aggarwal; All authors reviewed the results and approved the final version of the manuscript.

Ethical approval

Approval from the Institutional Ethics Committee was obtained prior to the commencement of the study (IEC approval code: 2018-10-EMP-102).

Patient consent

All patients/guardians provided written informed consent.

Data availability

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

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