Elevated serum IL-35 levels in rheumatoid arthritis are associated with disease activity

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ABSTRACT

To investigate serum interleukin (IL)- 35 levels in patients with rheumatoid arthritis (RA) and to describe the association between serum IL-35 levels and clinical parameters: erythrocyte sedimentation rate (ESR), C reactive protein (CRP), global health on Visual Analog Scale, Disease Activity Score in 28 joints based on ESR (DAS28-ESR), rheumatoid factor (RF) and anticyclic citrullinated peptide antibodies (ACPAs). The study included 129 patients with RA and 83 healthy controls. Serum IL-35 levels were detected by ELISA. ESR and CRP were measured by the Westergren method and the immune transmission turbidity method, respectively. RF and ACPA were measured using immunoturbidimetric assays and chemiluminescence analysis, respectively. The results showed that serum IL-35 levels were elevated in patients with RA. Univariate and multivariate analyses showed that the high serum IL-35 levels were correlated with low ESR and DAS28-ESR. These suggested that IL-35, an important anti-inflammatory cytokine, may participate in the regulation of the pathogenesis of RA, especially with disease activity.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease in which many cytokines including tumor necrosis factor (TNF)-α, interleukin (IL)−17 and IL-6 play a fundamental pathogenic role. RA is the most acknowledged inflammatory arthritis, with an estimated global prevalence in 2010 of 0.24%; it is about two times higher in women than men.[1] In recent years, IL-35, an anti-inflammatory cytokine, has been described to attenuate the symptom of collagen-induced arthritis (CIA), reduce incidence of CIA, prevent progression of bone damage, expand $CD4^+CD25^+$ regulatory T cells (Tregs), suppress $CD4^+CD25^-$ effector T cells and inhibit the differentiation of T helper cells (Th17 cells)/IL-17 cells in mice model.[2] Furthermore, in Murphy Roths Large/lpr mice model, IL-35 treatment could reduce the plasma levels of proinflammatory cytokines including IL-17, TNF-α and IL-6.[3]

IL-35, a member of the IL-12 cytokine family which includes IL-12, IL-23 and IL-27, is identified in 2007. All these four cytokines possess the chain-sharing properties. IL-35 is composed of an α chain (p35) shared with IL-12, and a β chain (Epstein-Barr virus induced 3(EBi3)) shared with IL-27.[4] However, distinct from other members in the IL-12 family, IL-35 is mainly produced by Tregs and directly involved in immunosuppression as an important effector molecule of Tregs. Besides, IL-35 could prevent the progression of autoimmune disease including experimental autoimmune encephalomyelitis and inflammatory bowel disease.[5 6] However, the role of IL-35 in human RA has not been clarified. At present, we investigated the serum IL-35 levels, as well as clinical features including disease duration, disease activity and autoantibody production in patients with RA.

MATERIALS AND METHODS

Patients

A total number of 129 patients who satisfied the 1987 American College of Rheumatology (ACR) and 2010 ACR/European League Against Rheumatism criteria[7 8] were recruited in this study (no prior use of calcium or vitamin D treatment, glucocorticoids or other bone active drugs). Patients were carefully excluded with definitive diagnoses other than RA. No one was treated with biological therapy. Demographic and general data, including age, gender and disease duration were collected at the same time. Blood tests for erythrocyte sedimentation rate (ESR) and C reactive protein (CRP) were measured by the Westergren method and the immune transmission turbidity method, respectively. Rheumatoid factor (RF) and anticyclic citrullinated peptide antibodies (ACPA) were measured using immunoturbidimetric assays (DiaSys Diagnostic Systems) and chemiluminescence analysis (Roche diagnostics), respectively. A positive result was identified as any titers >20 U/mL for RF and >17 U/mL for ACPA. Each patient's swollen joint count (SJC), tender joint count (TJC), global health on Visual Analog Scale (VAS) and Disease Activity Score in 28 joints based on ESR (DAS28-ESR) were assessed.[7] Written informed consent was provided by all subjects, and the study was conducted according to the Declaration of Helsinki.

Measurement of serum IL-35 levels

Blood samples were acquired from the patients with RA and healthy controls (HCs). Serum IL-35 levels were measured using ELISA kits

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(eBioscience, San Diego, California, USA) according to the manufacturer's protocol. Each sample was tested in duplicate. Between and within the assay, the coefficient of variation values were less than 15%. The optical density was measured at 450 nm using an automatic ELISA reader.

Statistical analysis

All data are presented as mean±SE or median (IQR) if continuous and as counts and per cent if categorical. Continuous variables from the study were analyzed by analysis of variance and/or Student's t-test with a parametric distribution or Mann-Whitney U test with a non-parametric distribution. Pearson's or Spearman's correlation coefficient was used to test the correlations between two variables. Multivariate linear regressions were used to analyze the covariates. All analyses were performed using SPSS V.17.0 (SPSS, Chicago, Illinois, USA), and GraphPad prism 6 software. Differences of $p<0.05$ were considered statistically significant.

RESULTS

Characteristics of the study population

The 129 patients with RA had a mean±SE age 54.1±3.6 years, while HCs had a mean±SE age 50.9±2.4 years. The disease duration with a median (IQR) was 6.9 (3.0–13.0) months. 95 (73.6%) patients were RF-positive and 69 (53.5%) patients were ACPA-positive. All patients with RA were treated with disease-modifying antirheumatic drugs (DMARDs) including: hydroxychloroquine, methotrexate, leflunomide and tripterygium glycosides (TGs). In DMARDs therapy, TG, a traditional Chinese herbal medicine, was used to treat RA. TG is an immunosuppressive element derived from the root of *Tripterygium wilfordii* which could attenuate the symptoms of RA and suppress cytokine production including IL-6, TNF-α and IL-8 in serum.[9 10]

Patients were later stratified into two groups of RA according to the 2015 ACR definition: early RA (<6 months duration) and established RA (≥6 months duration).[11] Expect for disease duration, there are no statistically significant differences in other clinical features between the 'early RA' group and the 'established RA' group (table 1).

Elevated serum IL-35 levels in patients with RA and its association with clinical parameters

The data were first analyzed in patients with RA and HCs. Serum IL-35 levels in patients with RA were higher than HCs (median (IQR), 6.3 (4.8–10.0) pg/mL vs 1.3 (0.7–2.5) pg/mL, $p<0.01$)(figure 1). The results showed that serum IL-35 levels were significantly increased in patients with established RA (median (IQR), 10.9 (6.0–19.1) pg/mL) compared with patients with early RA (median (IQR), 5.4 (4.3–7.2) pg/mL, $p<0.05$)(figure 1A).

High serum IL-35 levels were associated with low ESR ($r=-0.2$, $p=0.0251$), CRP ($r=-0.4$, $p<0.0001$), global health on VAS ($r=-0.2$, $p=0.0124$) and DAS28-ESR ($r=-0.2$, $p=0.0242$) (figure 1B–E). Additionally, serum IL-35 levels were lower in the RF-positive group (median (IQR), 6.0 (4.7–8.1) pg/mL) and the ACPA-positive group (median (IQR), 5.3 (4.5–6.9) pg/mL) compared with the RF-negative group (median (IQR), 10.9 (5.0–22.8) pg/mL) and the ACPA-negative group (median (IQR), 7.7 (5.4–14.1) pg/mL), respectively ($p<0.0001$, $p=0.0001$, respectively) (figure 1F). However, the correlation between IL-35 and other clinical features including TJC and SJC were not found in our study (data not shown).

Relationships among the main covariates were also evaluated statistically with multivariate linear regression.

Table 1 Clinical parameters of RA and HCs

Characteristics	Whole RA (n=129)	HCs (n=83)	P value	Patients with whole RA (n=129)		
				Early RA (n=85)	Established RA (n=44)	P value
Demographics						
Age, mean±SE, years	54.1±3.6	50.9±2.4	0.791	53.3±4.8	55.2±2.3	0.627
Sex (F/M)	113/16	71/12	0.210	72/13	41/3	0.423
Disease duration, median (IQR), months	6.9 (3.0–13.0)	–		3.1 (1.0–6.0)	11.4 (6.0–14.0)	0.006
Disease characteristics						
ESR, mean±SE, mm/hour	53.3±2.3	–		52.1±3.3	55.6±2.8	0.741
CRP, mean±SE, mg/L	41.8±4.4	–		39.8±7.2	43.5±2.9	0.912
TJC, mean±SE	7.0±1.6	–		7.0±2.8	7.0±1.2	0.871
SJC, mean±SE	8.0±1.4	–		7.0±1.1	9.0±2.0	0.793
Global health on VAS, mean±SE	7.0±0.2	–		7.8±0.1	6.0±0.7	0.629
DAS28-ESR, mean±SE	5.6±0.2	–		5.8±0.7	5.5±0.3	0.822
RF (+), n (%)	95 (73.6)	–		68 (80.0)	27 (61.4)	0.567
ACPA (+), n (%)	69 (53.5)	–		47 (55.3)	22 (25.9)	0.519
Medications						
HCQ, n (%)	46 (35.7)	–		30 (35.3)	16 (36.4)	0.912
MTX, n (%)	27 (20.9)	–		20 (23.5)	7 (15.9)	0.264
LEF, n (%)	39 (30.2)	–		13 (15.3)	26 (59.1)	0.183
TG, n (%)	36 (27.9)	–		18 (21.2)	18 (40.8)	0.622

ACPA, anticyclic citrullinated peptide antibody; CRP, C reaction protein; DAS28-ESR, Disease Activity Score in 28 joints based on erythrocyte sedimentation rate; ESR, erythrocyte sedimentation rate; HCQ, hydroxychloroquine; HCs, healthy controls; LEF, leflunomide; MTX, methotrexate; RA, rheumatoid arthritis; RF, rheumatoid factor; SJC, swollen joint count; TG, tripterygium glycosides; TJC, tender joint count; VAS, Visual Analog Scale.

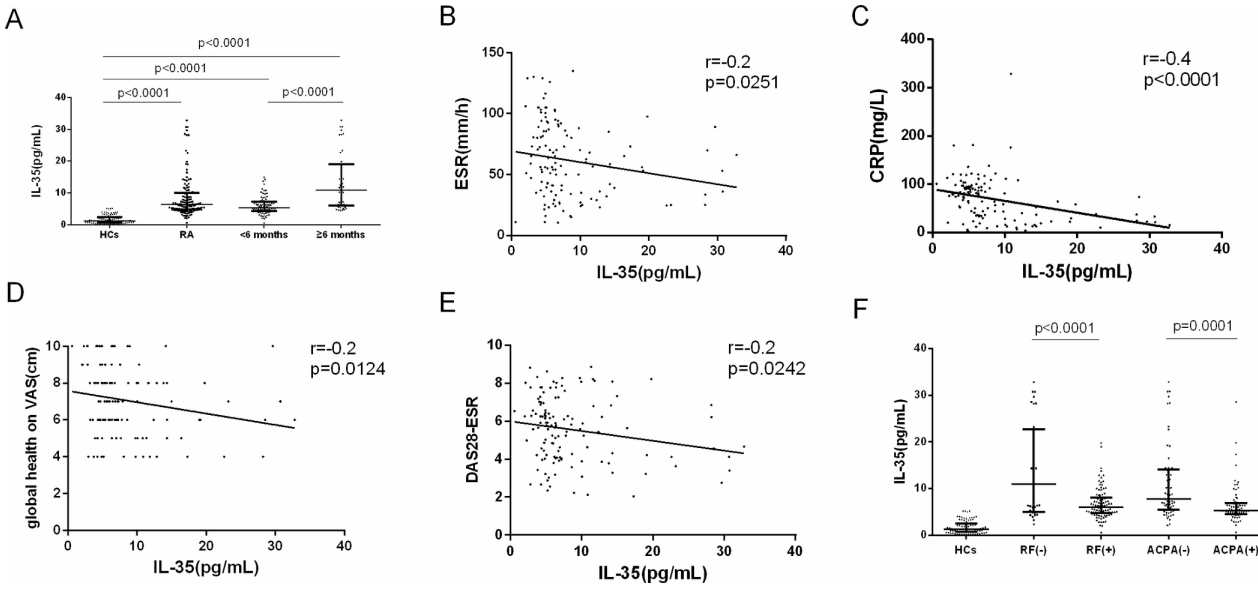

Figure 1 Serum IL-35 levels in patients with RA. (A) Serum IL-35 levels in patients with RA, HCs, patients with RA with disease duration <6 months and ≥6 months; (B,C,D,E) Associations between serum IL-35 levels and ESR, CRP, global health on VAS and DAS28-ESR; (F) Serum IL-35 levels in the RF/ACPA-positive group and the RF/ACPA-negative group. ACPA, anticyclic citrullinated peptide antibody; CRP, C reaction protein; DAS28-ESR, Disease Activity Score in 28 joints based on erythrocyte sedimentation rate; ESR, erythrocyte sedimentation rate; HCs, healthy controls; RA, rheumatoid arthritis; RF, rheumatoid factor; VAS, Visual Analog Scale.

Covariates considered for entry were gender, disease duration, menopausal status, ESR, CRP, SJC, TJC, global health on VAS, DAS28-ESR, RF, ACPA and medications. ESR and DAS28-ESR maintained significance in univariate and multivariate analyses, indicating serum IL-35 levels significantly associated with disease activity. In addition, multivariate linear regression analysis showed no associations of serum IL-35 levels with medical types in patients with RA (p>0.05) (table 2).

Table 2 Multivariate linear regression analysis of serum IL-35 levels

	OR	95% CI	P value
Gender	−0.140	−6.456 to 0.876	0.134
Disease duration	0.043	−0.096 to 0.166	0.599
Menopausal status	0.080	−1.377 to 3.509	0.389
ESR	−0.320	−0.073 to −0.019	0.001
CRP	−0.081	−0.054 to 0.019	0.064
TJC	−0.034	−0.235 to 0.307	0.079
SJC	−0.065	−0.231 to 0.390	0.061
Global health on VAS	−0.225	−1.370 to −0.224	0.070
DAS28-ESR	−0.208	−1.443 to −0.175	0.013
RF	−0.198	−2.439 to 0.016	0.061
ACPA	−0.113	−0.010 to 0.002	0.187
Medications			
HCQ	0.024	−2.655 to 3.306	0.829
MTX	0.131	−1.523 to 5.814	0.249
LEF	0.055	−3.142 to 4.724	0.691
TG	0.003	−3.544 to 3.470	0.983

ACPA, anticyclic citrullinated peptide antibodies; CRP, C reactive protein; DAS28-ESR, Disease Activity Score in 28 joints based on erythrocyte sedimentation rate; ESR, erythrocyte sedimentation rate; HCQ, hydroxychloroquine; LEF, leflunomide; MTX, methotrexate; RF, rheumatoid factor; SJC, swollen joint count; TG, tripterygium glycosides.; TJC, tender joint count; VAS, Visual Analog Scale.

DISCUSSION

Based on a small but very homogeneous sample of recruited patients, we found that serum IL-35 levels were significantly higher in patients with RA which coincided exactly with experimental results of Filková M *et al* and Ladislav Šenolt *et al*.[12 13] We could deduce that the endogenous elevation of IL-35 may participate in the negative feedback immune response activated by inflammatory factors, which could antagonize excessive inflammatory response, and finally play a protective role in patients with RA. Given the current and previous studies, IL-35 may play an immunoregulatory role in the inflammatory milieu of RA.

Our results show that high serum IL-35 levels are significantly associated with low disease activity. We hypothesized these associations were partly dependent on inflammatory cytokines including IL-17, TNF-α, IL-6, and so on, under high disease status. Furthermore, in multivariate liner regression analyses, the relationships between serum IL-35 levels and ESR and DAS28-ESR were not changed. This association remained significant after adjustment suggesting a significant effect of IL-35 on disease activity in patients with RA. Meanwhile, IL-35 is vitally correlated with disease activity in other autoimmune diseases such as systemic lupus erythematosus (SLE) and inflammatory bowel diseases. In patients with active SLE, serum IL-35 levels were increased with a low soluble level of IL-35 receptors on $CD4^+$ Th.[14] In patients with active ulcerative colitis, IL-35 (EBi3) mRNA levels in colonic mucosa were significantly upregulated compared with patients with inactive ulcerative colitis.[15] Taken together, these results may suggest that IL-35 has an important role in the pathogenesis of disease activity in RA.

We also observed that the long disease duration contributed to significant increase of serum IL-35 levels suggesting that IL-35 may play different roles at different phases of

RA. However, the specific mechanism still needs to be investigated. Furthermore, we could also hypothesize that the high serum IL-35 levels might not be sufficient to induce the augmentation of Tregs in patients with early RA, indicating that IL-35 might not respond well to the autoimmune disease without a high level of expression of Tregs. Additionally, reduced serum IL-35 levels appeared to be related with autoantibody production. IL-35, also secreted by activated B cells to a lesser extent, was shown to have an important impact on autoantibody production.[16] This finding indicated that IL-35 was potentially associated with the immunopathology of RA.

Together, our results suggested that IL-35 in RA correlated with disease activity. Studies have shown the correlation between RA pathogenesis and Th17/IL-17.[17] IL-35 could suppress differentiation of Th17/IL-17 cells.[2] On this basis, it was hypothesized that IL-35 could potentially have an effect on RA via suppression of the Th17/IL-17-related pathway. Further studies on molecular mechanisms to substantiate these assumptions are warranted.

CONCLUSIONS

In conclusion, serum IL-35 levels were increased in patients with RA and were associated with disease activity, indicating a possible therapeutic significance for IL-35 in RA.

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