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Decreased Gene Expression of Epstein–Barr Virus-Induced Gene 3 (EBI-3) may Contribute to the Pathogenesis of Rheumatoid Arthritis

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ABSTRACT

Background: Interleukin-35 (IL-35) is a member of the IL-12 family of heterodimeric cytokines produced by regulatory T (Treg) cells. This immunosuppressive cytokine can prevent exaggerated inflammatory responses like those responsible for the development of rheumatoid arthritis (RA). This study aims to determine the correlation between the gene expression of Epstein–Barr virus-induced gene 3 (EBI-3) and IL-12A (p35) subunits of IL-35 in peripheral blood leukocytes with immunological and clinical parameters in RA patients.

Methods: We recruited 47 patients with RA and 44 healthy subjects. The disease activity score-28 (DAS-28) was assessed by an expert rheumatologist and the plasma levels of neopterin and anti-cyclic citrullinated peptide (anti-CCP) was measured using ELISA method also Serum rheumatoid factor (RF) was assessed by the agglutination test. For the evaluation of IL-12A and EBI-3 gene expression, we used qPCR.

Results: We did not find any significant correlation between the gene expression of IL-35 subunits and DAS-28. There was a negative correlation between the plasma levels of neopterin and the gene expression of EBI-3 ($p = 0.004$). Inversely, we found a positive correlation between plasma level of anti-CCP and neopterin ($p < 0.001$) also between RF and DAS-28 ($p = 0.001$).

Conclusion: Regarding the significant negative correlation between EBI-3 gene expression and plasma levels of neopterin, it can be concluded that the altered gene expression of EBI-3 may play a role in the pathogenesis of RA.

KEYWORDS
Rheumatoid arthritis; interleukin-35; neopterin; DAS-28; FoxP3

Introduction

Effector T cells including, Th17 and Th1 cells have a crucial role in both induction and maintenance of rheumatoid arthritis (RA) pathological features (Alzabin and Williams, 2011). Regulatory T (Tregs) cells efficiently contribute to the immune system homeostasis (Sakaguchi et al., 2008). Tregs express a transcription factor called Forkhead box protein (FoxP3) and secrete inhibitory cytokines such as interleukin-10 (IL-10) and transforming

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growth factor-β (TGF-β) (Fontenot et al., 2003). IL-35 is a new cytokine and its anti-inflammatory function is described recently (Collison et al., 2010). It is produced by a different cell type including regulatory and conventional T cells in mice and human (Chaturvedi et al., 2011). IL-35 belongs to the IL-12 family of heterodimeric cytokines and consists of two distinct subunits, EBI-3 (Epstein–Barr virus-induced gene 3) and IL-12A (p35) (Trinchieri et al., 2003). The other members of this family including IL-12 (p40, p35), IL-23 (p40, p19), and IL-27 (EBI-3, p28) all have two subunits and expression of both of them is essential for the secretion of the biological active cytokine. While both IL-35 and IL-27 play an immunosuppressive function, the two other members of this family have inflammatory characteristics (Aparicio-Siegmund and Garbers, 2015; Olson et al., 2013). Activated monocytes/macrophages and dendritic cells (DC) are the principal cellular sources of IL-27, IL-23, and IL-12 (Sawant et al., 2015). Also, IL-27 is produced by astrocytes and microglia in the nervous system (Pan et al., 2010). The effect of IL-27 on fibroblast-like synoviocytes and inflammation of joint in RA has been documented (Wong et al., 2010). EBI-3 have relation with the p40 subunit of IL-12 and can antagonize its effects in provoking Th1 responses (Ollier, 2000) (Ehrenstein. et al., 2004, Ueda et al., 2003). Failure of self-tolerance results in accumulation of immunological and inflammatory mediators such as C-reactive protein (CRP), rheumatoid factor (RF), anti-cyclic citrullinated peptide (anti-CCP) in synovial fluid and serum of RA patients (Amos et al., 1977; Kastbom et al., 2004; Luthra et al., 1975). Neopterin is among well-recognized immunological mediators which its production increases in RA patients (Reibnegger et al., 1986). It is a pyrazino-pyrimidine compound produced following catabolism of guanosine triphosphate (GTP) and its levels in the body fluids reflects the intensity of cell-mediated immunity activation (Beckham et al., 1992). Neopterin is produced following the activation of cell-mediated immunity by interferon-γ (IFN-γ) (Akgul et al., 2013). The assessment of neopterin levels could be used to follow up the treatment process in RA patients also its level may predict disease activity and severity (D'agostino et al., 2013). Furthermore, there is a correlation between neopterin levels and radiographic severity in osteoarthritis patients (Zhou et al., 2013); however, neopterin can be helpful as a non-specific biomarker for diagnosis of RA (Arshadi et al., 2013; Shady et al., 2015). Since both neopterin and IL-27 are produced by macrophages and monocytes in the immune system, there may be a connection between their biosynthetic processes (Guzzo et al., 2012). The purpose of this study was to evaluate the correlation between the gene expression of IL-35 subunits and FoxP3 in peripheral blood leukocytes with immunological and clinical parameters of RA disease including anti-CCP, RF, CRP, neopterin, and disease activity score-28 (DAS-28).

Material and methods

Study design and participation

We conducted a cross-sectional study and took a consecutive sample of RA patients attending Helal Ahmar clinic of the Kermanshah University of Medical Sciences (KUMS). The study group included 47 patients (40 women and 7 men, mean age: 50.81 ± 12.19 years) diagnosed with RA based on the 2010 American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) criteria and

44 sex- and age-matched healthy subjects. The following laboratory and clinical parameters were measured in our participants: RF, anti-CCP, erythrocyte sedimentation rate (ESR), CRP and DAS-28 based on ESR (DAS28-ESR). Based on ESR concentration, a disease activity was assessed by the evaluation of the DAS-28. The DAS-ESR score is a combination of information from the 28 tender and swollen joints, the ESR, and the patient's visual analog scale (VAS). VAS is the patient's point of view about the severity of illness, which is considered to be between 0 and 100, with 0 the best condition and 100 the worst one (Nielung et al., 2015). RA disease activity can be interpreted as remission (DAS28-ESR < 2.6), low (DAS28-ESR 2.6–3.2), moderate (DAS28-ESR 3.2–5.1), and high (DAS28-ESR > 5.1). Patients with moderate and high DAS considered as having active RA, whereas those with low disease activity had inactive RA (5). Our patients with active RA received combinational disease modifying anti-rheumatic drug (DMARD) including methotrexate (range from 7.5 to 25 mg/week), and hydroxychloroquine (200 mg/day) plus oral prednisolone (range from 5 to 10 mg/day). We excluded patients from our study if (1) they were pregnant; (2) had used biologic DMARD; or (3) had other concomitant autoimmune diseases. Also according to the declaration of Helsinki and the ethical committee of KUMS, we obtained written informed consent from all participants.

Blood sample collection

About 5 ml peripheral blood was taken from patients and healthy group. Plasma was separated in (ethylene diamine tetra acetic acid) EDTA containing tubes and centrifuged at 3000 g for 10 min and was kept in −70°C until analysis.

ELISA and agglutination test

Plasma levels of neopterin and anti-CCP was measured using ELISA kit IBL, Germany (ref. RE59321 – lots ENO221) and Euro immune company (order no. EA1505-9601 G, lots E160504BT), respectively. Also, plasma levels of RF were measured with latex agglutination test and ESR was obtained from the patient's records.

RNA isolation and cDNA synthesis

RNA was extracted from peripheral blood mononuclear cells, according to the manufacturer's instruction (CinnaGen Inc., Cat No.: RN7713C, Tehran, Iran). Quality and quantity of RNA were measured by Nanodrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Waltham Massachusetts, USA) and the purity was assessed by 1% agarose gel electrophoresis. For this purpose, the extracted RNA was checked for ribosomal RNA (rRNA) 28S and 18S bands. The intensity of the upper ribosomal band (28S) was about twice the intensity of the lower band (18S). The extracted RNA was aliquoted and kept at −70°C until use. The cDNA synthesis was performed on 300–500 ng of RNA in a 20 volume using Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland) as recommended by the manufacturer; finally, all cDNA samples were kept at −70°C until further analysis.

Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed using the LightCycler 96® Instrument (Roche Applied Science, Penzberg, Germany) on the following program including, preincubation, 30 s, 95°C 45 cycle, 3 step amplifications (5 s at 95°C, 30 s at 62°C, 30 s at 72°C 45 cycles, melting 5 s at 95°C, 60 s at 60°C, 1 s at 95°C and continuous melting. Also, this reaction was performed in a total volume of 20 µL including 2 µL cDNA, 0.5 µL forward primer (0.5 mM), 0.5 µL reverse primer (0.5 mM), 7.5 µL of SYBER® Premix Ex Taq™ II (Takara, Japan) and 9.5 µL nuclease-free water.

Gene expression of EBI-3, IL-12A, and FoxP3 was normalized relative to the β-actin as a housekeeping gene. The quantity of mRNA (relative quantification, RQ) was given as $2^{-\Delta\Delta ct}$. ΔΔct was calculated as followed: ΔΔct = Δct (patient) − Δct (control). Δct = ct (target gene) − ct (endogenous control). Specific primer pairs for each gene were designed using online software (Oligocalc and Oligoanalyzer). Accuracy and specificity of primers were checked using the Basic Local Alignment Search Tool on the US National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The sequences of the human primers used are shown in Table 1.

Statistical analysis

Statistical analysis was performed using SPSS, version 18 (Inc., Chicago, IL, USA). The data were analyzed using descriptive and inferential statistics. In the descriptive statistics, central orientation and dispersion criteria along with charts and diagram were reported. The data normality was assessed by the Kolmogorov–Smirnov (KS) test. The T-test and Mann–Whitney test were used for parametric and non-parametric data, respectively. Pearson correlation coefficient and the Chi-square test were used to investigate the relationship between quantitative and qualitative variables, respectively. The significance level was considered ($P < 0.05$).

Results

The comparison of the study variables in patient and control groups was depicted in Table 2. The correlation between gene expression of Foxp3, EBI-3, and IL-12A with neopterin and DAS-28 is shown in Table 3. As depicted in Table 2, the plasma level of neopterin was significantly higher in RA patients in comparison with healthy controls ($p = 0.038$). In contrast, the expression of FoxP3 mRNA and EBI-3 subunit was down-regulated significantly in RA patients ($p < 0.001$), inversely the gene expression of IL-12A, the other IL-35 subunit was increased significantly in RA patients ($p < 0.001$). A significant correlation between anti-CCP and neopterin ($p < 0.001$), RF and DAS-28 ($p = 0.001$) was observed in our study.

Table 1. Primer designed for housekeeping gene and the gene of interest.

Gene name	Forward primer sequences	Reverse primer sequences
β-Actin	AATGAGCTGCGTGTGGCTCCC	CAGGGATAGCACAGCCTGGATAGCA
FoxP3	AGCACATTCCCAGAGTTCCTC	CGTGTGAACCAGTGGTAGATC
EBI-3	TACGTGCTCAATGTCACCG	GGCTTGATGATGTGCTCTGTTA
IL-12A	CATAACTAATGGGAGTTGCCTGG	TAACTGCCAGCATGTTTTGATCTAG

Table 2. Comparison of the variables studied in patient and control groups.

Variables	Control	Patients (RA)	p-Value
Age	46.34 ± 8.88	50.81 ± 12.19	0.054[a]
Sex			
Male	7 (16.7%)	7 (14.9%)	0.053[b]
Female	35 (83.3%)	40 (85.1%)	
RF	0 (0–1)	1 (1–3)	<0.001[c]
Anti-CCP	5.074 ± 24.530	82.687 ± 85.641	<0.001[a]
Neopterin	15.32 ± 9.02	19.73 ± 9.68	0.038[a]
FoxP3	1.000 ± 0.000	0.215 ± 0.282	<0.001[a]
EBI-3	1.000 ± 0.000	0.496 ± 0.372	<0.001[a]
IL-12A (p35)	1.000 ± 0.000	2.387 ± 1.570	<0.001[a]

SD: standard deviation, mean ± standard deviation, median (interquartile range), and percentage (%) are presented for parametric, non-parametric, and categorical data, respectively.
[a]Independent sample T-test,
[b]Chi-square test,
[c]Mann–Whitney U.

Table 3. Correlation between clinical and laboratory characteristic.

Variable	DAS-28	Neopterin
ESR	r 0.723[a]	r 0.055[a]
	p <0.001	p 0.716
RF	r 0.455[b]	r 0.165[b]
	p 0.001	p 0.273
Anti-CCP	r 0.015[b]	r −0.510[b]
	p 0.922	p <0.001
FoxP3	r −0.123[b]	r −0.150[b]
	p 0.457	p 0.200
EBI-3	r −0.190[b]	r −0.327[b]
	p 0.234	p 0.004
IL-12A (p35)	r −0.078[b]	r 0.099[b]
	p 0.634	p 0.394

[a]Pearson correlation,
[b]Spearman correlation.

Table 4. Univariate and multivariate regression model for association between neopterin and other variables.

	Univariate				Multivariate			
		95.0% Confidence interval for β				95.0% Confidence interval for β		
	β	Lower bound	Upper bound	p-Value	β	Lower bound	Upper bound	p-Value
Anti-CCP	−.022	−.049	.005	.113	−.051	−.083	−.019	**.002**
FoxP3	−3.213	−8.161	1.735	.200	−4.940	−11.857	1.976	.159
EBI-3	−8.416	−14.002	−2.829	**.004**	−9.843	−16.560	−3.126	**.005**
IL12-A	.712	−.941	2.364	.394	−.784	−3.026	1.457	.488

Correlation between clinical and laboratory characteristic was shown in Table 3. Also for confirmation of the correlation of neopterin with other variables, multivariate analysis was performed (Table 4).

After calculation of DAS-28 for each patient according to the formula (Nielung et al., 2015):

- DAS28-CRP = 0.56 .√TJC28 + 0.28 .√SJC28 + 0.014. PtGH + 0.36. ln(CRP) + 0.96.
- DAS28-ESR = 0.56 .√TJC28 + 0.28 .√SJC28 + 0.014 .PtGH + 0.70. ln(ESR).

We could not find any significant differences in the mRNA expression of IL-35 subunits and FoxP3 genes between patients with low, moderate, and high disease activity and patients with DAS-28 in remission limits (Figure 1). As it shown in Table 3, we could not find any significant correlation between the gene expression of IL-35 subunits and DAS-28. This is also being true for the FoxP3 gene expression and DAS-28. Contrarily, significant correlation was seen between the EBI-3 gene expression and the plasma levels of neopterin ($p = 0.04$). The correlation between DAS-28 with neopterin ($p = 0.124$), EBI-3 ($p = 0.234$), and IL-12A ($p = 0.634$) was shown (Figures 2, 3 and 4).

Discussion

The currently available data regarding the role of IL-35 in the pathogenesis of RA is controversial (Filková et al., 2015; Nakano et al., 2015). In the present study, we surveyed the gene expression of IL-35 subunits in peripheral blood leukocytes of RA patients to evaluate its association with clinical and laboratory parameters. Interestingly, the gene expression of EBI-3 subunit decreased while the other subunit, IL-12A showed elevated gene expression in our patients. EBI-3 is a subunit of two different cytokines: IL-35 and IL-27 (Yoshida et al., 2009). IL-35 has a clear-cut anti-inflammatory function while both pro- and anti-inflammatory properties have been described for IL-27 in the previous studies (Villarino et al., 2004). Since both IL-35 and IL-27 share EBI-3 subunit, the low expression of EBI-3 in peripheral blood leukocyte of RA patients may disrupt the production of these two cytokines and result in defective immune regulation mechanisms in RA patients. Our result is compatible with a recent finding which showed a low level of IL-35 protein in the plasma of RA patients (Ning et al., 2015). Unlike this finding, the results of other study showed that the plasma levels of IL-35 increases at the onset of RA and reduces following the initiation of treatment (Šenolt et al., 2015). IL-12A is another

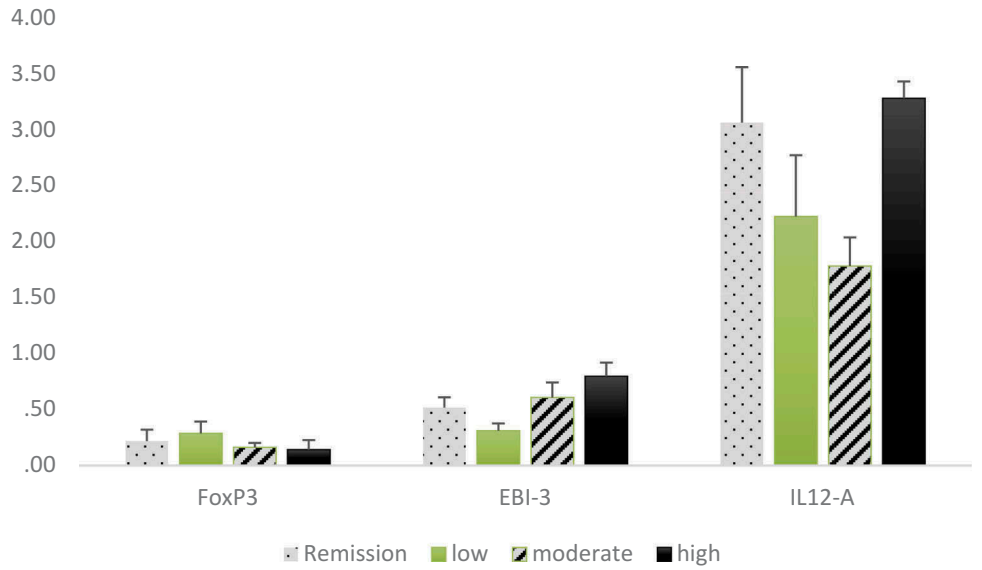

Figure 1. The mRNA expression of FoxP3, EBI-3, and IL-12A based on the DAS-28 grade.

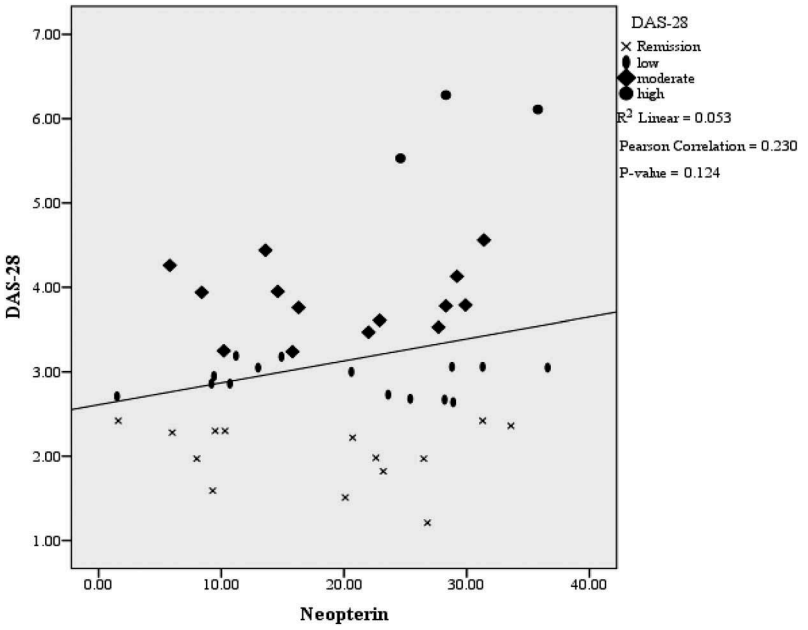

Figure 2. The correlation between DAS-28 and neopterin level.

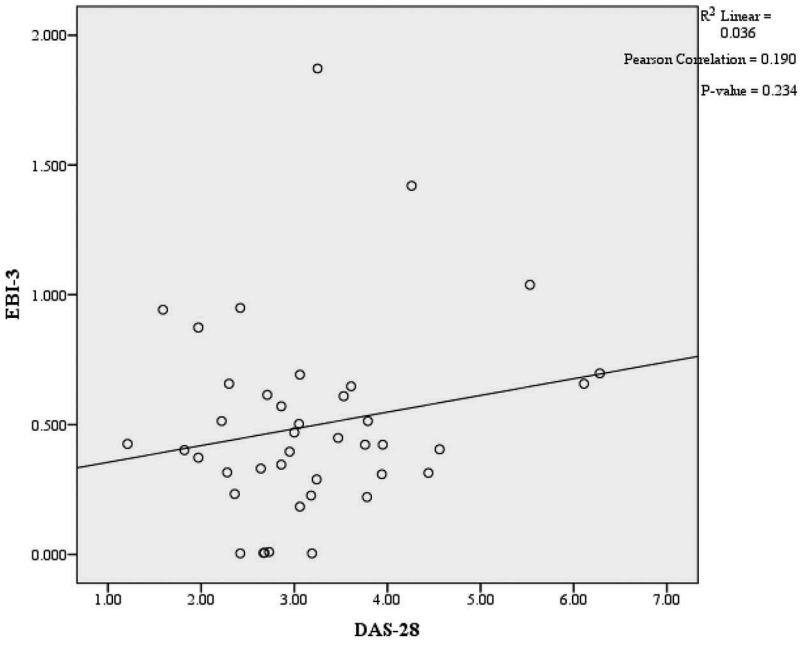

Figure 3. The correlation between DAS-28 and EBI-3 expression.

subunit of IL-35, shared with IL-12, the increased IL-12A subunit gene expression that we observed in our study may cause overproduction of IL-12, a key player cytokine in cell-mediated immunity. Compatible with our finding, the elevated levels of IL-12 have been

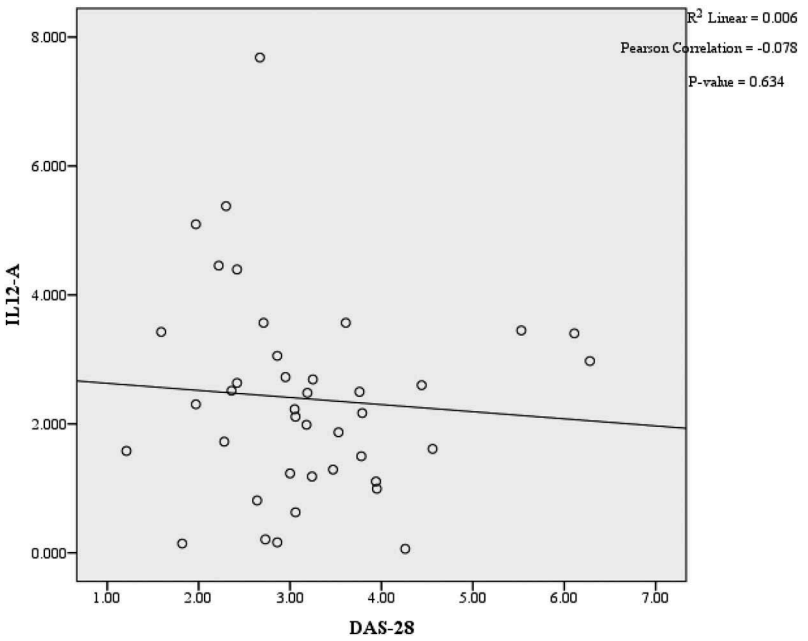

Figure 4. The correlation between DAS-28 and IL-12A expression.

reported in RA patients in the previous study (Petrovic-Rackov and Pejnovic, 2006). In the following, for clarification of the effects of altered EBI-3 and IL-12A gene expression on a clinical parameter, we analyzed their correlation with DAS-28, a well-accepted instrument for monitoring disease activity in RA patients (Prevoo et al., 1995). There was not any significant association between DAS-28 and IL-35 subunits gene expression in patients in remission group and also in patients with high, low, and moderate disease activity. Contrary to our finding, synovial fluid analysis of RA patients in another study showed a positive association between IL-35 and disease activity (Šenolt et al., 2015). The reason for this controversy is attributable to the ectopic production of IL-35 in synovial fluid which might be different from its systemic production in the peripheral blood (Collison et al., 2010). In contrast to TGF-β, IL-35 is an anti-inflammatory cytokine that its expression increases in the inflammatory milieu which is the characteristic of inflamed synovium (Li et al., 2012). Along with these findings, ex-vivo results showed both EBI-3 and IL-12A messenger RNA are markedly up-regulated in Treg cells co-cultured with effector T cells (Collison et al., 2010). In the following, we also survey the gene expression of FoxP3, another critical molecule in the function of Treg cells to define its correlation with DAS-28 and disease biomarkers. The previous study showed that the expression of EBI-3 is dependent on the FoxP3 transcription factor. EBI-3 is a downstream target of Foxp3, a transcription factor that is required for Treg cell development and function (Collison et al., 2007). Like EBI-3, the gene expression of FoxP3 was significantly lower in RA patients in comparison with healthy subjects, which was compatible with our previous study that showed increased methylation and reduced gene expression of Foxp3 locus in patients with RA (Zafari et al., 2018). Collectively, these data imply compromised effector function of Treg cells is in RA patients. In the following, we analyzed the relationship

between RA immunological and inflammatory indicators including anti-CCP, RF, CRP, ESR and neopterin with the gene expression of EBI-3, IL-12A, and FoxP3. We could not find any significant correlation between these variables except for neopterin which had a negative correlation with EBI-3 gene expression and positive correlation with anti-CCP. It can be concluded that anti-CCP antibody production may increase simultaneously with inflammatory markers like neopterin in RA patients (Arshadi et al., 2013). Positive anti-CCP antibody could be a risk factor for a more aggressive form of RA (Del Amo et al., 2006). The increased neopterin plasma levels reflect exaggerated macrophage activation which is mediated by cytokine IFN-γ (Fuchs et al., 1988). Activation of macrophages is a hallmark of inflammatory disease such as RA (Kinne et al., 2000). In our survey, plasma levels of neopterin were significantly higher in RA patients compared to healthy subjects. The negative correlation between neopterin levels and EBI-3 subunits in our survey may reflect impaired Treg effector function in controlling of cell-mediated immunity (CMI) which leads to elevation of plasma neopterin levels. Our finding is supported by the previous animal study which showed exaggerated CMI in the EBI-3 knockout mice (Tong et al., 2010).

Conclusion

Briefly, our results supported the previous studies which showed impairment of IL-35 production in RA patients. Furthermore, we brought up the possible role of EBI-3 subunit of IL-35 gene expression in the pathogenesis of RA which needs to be analyzed meticulously.

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Disclosure statement

The authors declare no conflict of interest and the authors are responsible for the content and writing of the paper.

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