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Overexpression of interferon-γ and indoleamine 2, 3-dioxygenase in systemic lupus erythematosus: relationship with the disease activity

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Abstract

Background: Indoleamine 2, 3-dioxygenase (IDO) is a tryptophan catabolizing enzyme which is involved in immune regulation and autoimmune disorders such as systemic lupus erythematosus (SLE). Interferon-γ (IFN-γ) is an inflammatory cytokine which is the major inducer of IDO expression. Here, we evaluated the level of IFN-γ and IDO among SLE patients in correlation with the severity of SLE.

Methods: Fifty-three SLE patients and 35 age matched healthy donors were enrolled in this study. Systemic lupus erythematosus disease activity index (SLEDAI) was used to calculate the disease activity. Real-time RT-PCR and ELISA were used to evaluate the gene expression of IDO and IFN-γ plasma concentration, respectively.

Results: We showed that IDO-1, IDO-2 and IFN-γ were over-expressed among SLE patients significantly (p < 0.0001). There were significant positive correlations between IFN-γ with the expression of IDO-1 (r = 0.722, p < 0.0001) and IDO-2 (r = 0.682, p < 0.0001). There were also positive correlations between SLEDAI scores with IDO-1 (r = 0.675, p < 0.0001), IDO-2 (r = 0.727, p < 0.0001) and IFN-γ (r = 0.907, p < 0.0001).

Conclusions: IDO expression and IFN-γ level could be introduced as helpful biomarkers for the determination of disease severity in SLE patients.

Keywords: biomarker; indoleamine 2, 3-dioxygenase; interferon-γ; SLEDAI; systemic lupus erythematosus.

Introduction

Systemic lupus erythematosus (SLE) as a multifactorial autoimmune disease is associated with various clinical manifestations by targeting multiple organ including skin, joints, blood cells, kidneys, and central nervous system [1, 2]. Although genetic susceptibility, environmental triggers and hormonal condition in addition to viral infections are proposed to be involved in pathophysiology [3], the underlying mechanism of the onset and propagation of SLE is not well elucidated. According to the heterogeneity of SLE, implementation of current treatment strategies solely rely on SLE disease activity scores derived from clinical manifestations and nonspecific laboratory analyses [2, 4]. Although most of the clinicians use complement and anti-dsDNA levels, lack of reliable instrumental or laboratory tests is a major issue to determine disease activity among SLE patients [5]. Characterization of disease activity by means of a reliable and reproducible laboratory tests could be helpful in describing aspects of disease, predict prognosis and determining therapeutic approaches.

Interferon-γ (IFN-γ) is a key cytokine which controls Th1-dependent immune responses [6]. It plays a critical role during the development of autoimmunity, especially in SLE. The over-production of IFN-γ is believed to be correlated with the SLE pathogenesis and disease activity [7]. However, IFN-γ is not routinely measured to assess the disease severity among SLE patients.

Indoleamine 2, 3-dioxygenase (IDO) is an intracellular heme-containing dioxygenase catabolizing tryptophan which is an initial and rate-limiting enzyme of kynurenine pathway [8]. Tryptophan degradation leads to the inhibition of surrounding T cells function [9]. IDO is generally expressed in antigen-presenting cells, macrophages and dendritic cells. Inflammatory cytokines, in particular IFN-γ, are known as main inducers of IDO expression [10].
Accordingly, IDO plays a crucial role in the induction of immune tolerance and immune response regulation [11]. Increased IDO activity is observed in many Th1-mediated autoimmune diseases such as SLE [12]. Although these findings indicate that IDO is upregulated in the plasma and peripheral blood mononuclear cells (PBMCs) of SLE patients in accordance with disease activity [13, 14], lack of reliable biomarkers and monitoring methods are still major issues. Moreover, the imbalances in the production of IFN-γ-related genes are considered to be involved in the development of various clinical features seen in SLE [15]. Thus, detection of IFN-γ and related downstream molecules could be helpful in monitoring SLE and could be proposed as markers for the disease activity. Therefore, we evaluated the production of IFN-γ and mRNA expression of IDO-1 and IDO-2 among SLE patients to address the correlation of these factors with the severity and progression of SLE.

Materials and methods

Patients and controls

A total of 53 female patients (29 under treatment and 24 newly diagnosed) fulfilling 4 out of 11 items of the revised American College of Rheumatology criteria for SLE [2] were enrolled in this study at Sayyad Shirazi educational hospital, rheumatology department, Golestan University of Medical Sciences. Thirty-five age and sex matched healthy donors were also recruited. All patients with an active infection, or inflammation, pregnancy and/or history of other autoimmune disorders were excluded. The disease activity was calculated in SLE patients by systemic lupus erythematosus disease activity index (SLEDAI) [2]. Accordingly, SLEDAI scores lower than or equal to 10 were considered as low and scores greater than 10 represented high disease activity [16]. Written informed consent was obtained from all patients following the Declaration of Helsinki [17]. This study was approved by the Ethical Committee of Golestan University of Medical Sciences. A volume of 5 mL whole blood were taken from all subjects and plasma were separated. Isolated plasma were stored at – 80 °C for the measurement of IFN-γ. PBMCs were also isolated using Ficoll-Paque (Baharafshan, Tehran, Iran) density-gradient centrifugation, as described elsewhere [18].

RNA isolation and reverse transcription-real time quantitative PCR analysis

PBMCs were used for the isolation of total RNA with Biozol (Bioer, China) according to the manufacturer’s protocol. Using Bioer cDNA synthesis kit (BioFlux, Bioer, China) 1 μg of total RNA was reverse transcribed to cDNA with random hexamer primers and also treated with DNase I (Sinaclon, Iran) to remove possible genomic DNA contaminations. Real-time RT-PCR was performed with Bioer detection system (Bioer). 18s ribosomal RNA (18s rRNA) was used as a suitable internal control for gene expression normalization. Gene specific primers for IDO-1, IDO-2 and 18srRNA are summarized in Table 1. All primers were designed and evaluated to span exon-exon junctions. PCR amplifications were performed using Bioer SYBR green qPCR master mix (BioFlux).

Quantification of IFN-γ plasma levels using ELISA

The plasma level of IFN-γ in SLE patients and healthy donors were determined using commercially available ELISA kit (Biolegend, CA, USA) according to the manufacturer’s instructions. Biotek ELISA reader ELX800 (Biotek, VT, USA) was used to obtain the optical density of each sample at the wavelength of 450 nm. All samples were assayed in triplicates and the results were reported as picograms per mL (pg/mL).

Statistical analyses

Data are presented as means±SD (standard deviation). All of the experiments for each sample were repeated in triplicates. Statistical software SPSS 22.0 and Graphpad Prism 5.04 were used for data analysis and graphs preparation. The nonparametric Kruskal-Wallis test was used for comparing means of multiple samples. Mann-Whitney U-test was used to compare differences between two groups. Correlation analyses were performed using two-tailed Spearman’s rank correlation. Positive r-values represent a positive correlation while negative r-values represent a negative correlation. p-Values lower than 0.05 were considered as statistically significant.

Table 1: Gene specific primers used for real time RT-PCR.

<table>
<thead>
<tr>
<th>Primer (accession)</th>
<th>Sequence (5′&gt;3′)</th>
<th>Tm</th>
<th>Amplicon size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDO-1 (NM_002164)</td>
<td>F: CTACCATCTGCAAATCGTGACTAAGT</td>
<td>60</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>R: GAAGGGTCTTCAGAGGTCTTATTCTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IDO-2 (NM_194294)</td>
<td>F: GCCACGAATGCTATCTTG</td>
<td>60</td>
<td>169</td>
</tr>
<tr>
<td></td>
<td>R: TGTCTTTCCATCCAGAGAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18srRNA (M10098)</td>
<td>F: CAGCCACCCCGAGATTGAGCA</td>
<td>61</td>
<td>252</td>
</tr>
<tr>
<td></td>
<td>R: TAGTAGCGACGGCGGTGTG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Results

Clinical and laboratory characteristics

The demographic and clinical characteristics of SLE patients and subsequent statistical results are illustrated in Table 2. In order to determine the disease activity, SLEDAI score was applied.

Overexpression of IDO-1, IDO-2 among SLE patients

Real time RT-PCR data showed that the mRNA expression level of IDO-1 and IDO-2 in the PBMCs of newly diagnosed SLE patients is significantly higher than the patients receiving treatments (p<0.0001) and normal subjects (p<0.0001) (Figure 1A and B). In order to express data relatively, the mean mRNA levels in healthy donors were arbitrarily set at the fold change of 1.

The level of IFN-γ is increased among newly diagnosed SLE patients

We also assessed the plasma level of IFN-γ using ELISA method. As shown in Figure 1C, there is no significant difference between the level of IFN-γ among healthy donors and under treatment SLE patients. However, the level of IFN-γ is significantly higher among newly diagnosed SLE patients (p<0.0001).

Correlation analyses between IDO-1, IDO-2, IFN-γ and SLE severity

Spearman correlation study was performed to evaluate the correlation between expression of IDO-1, IDO-2 and the plasma level of IFN-γ in the PBMCs of SLE patients. As shown in Figure 2A, there was a significant positive correlation between IDO-1 mRNA expression and IFN-γ level (r=0.722, p<0.0001). Similarly, a significant positive correlation was observed between IDO-2 mRNA expression and IFN-γ level (r=0.682, p<0.0001) (Figure 2B).

To evaluate together the relationship between IDO-1, IDO-2 and IFN-γ and the severity of SLE, a correlation among these markers and SLEDAI scores was carried out. As shown in Figure 2C, there is a significant positive correlation between IFN-γ plasma levels and SLEDAI scores (r=0.907, p<0.0001). Furthermore, IDO-1 mRNA expression levels are positively correlated with the SLEDAI scores (Figure 2D) of SLE patients significantly (r=0.675, p<0.0001). There is also a significant positive

Table 2: Demographic characteristics, clinical manifestations, and laboratory parameters of patients with systemic lupus erythematosus.

<table>
<thead>
<tr>
<th>Characteristic (n=53)</th>
<th>IFN-γ (p-value)</th>
<th>IDO-1 (p-value)</th>
<th>IDO-2 (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>29.17±9.83</td>
<td>38.18±33.74</td>
<td>7.46±3.59</td>
</tr>
<tr>
<td></td>
<td>(r=0.187, p=0.181)</td>
<td>(r=0.187, p=0.181)</td>
<td>(r=0.232, p=0.95)</td>
</tr>
<tr>
<td>Disease duration</td>
<td>2.17±2.91</td>
<td>13.98±15.1</td>
<td>6.20±3.77</td>
</tr>
<tr>
<td></td>
<td>(r=−0.494, p&lt;0.0001)</td>
<td>(r=0.907, p&lt;0.0001)</td>
<td>(r=0.675, p&lt;0.0001)</td>
</tr>
<tr>
<td>Mean SLEDAI</td>
<td>26 (49.1)*</td>
<td>26 (49.1)*</td>
<td>26 (49.1)*</td>
</tr>
<tr>
<td>Low disease activity (SLEDAI ≤10)</td>
<td>8.28±4.48 (&lt;0.0001)</td>
<td>6.10±2.70 (0.663)</td>
<td>2.80±0.88 (0.023)</td>
</tr>
<tr>
<td>High disease activity (SLEDAI &gt;10)</td>
<td>60.19±39.79</td>
<td>8.90±3.90</td>
<td>3.81±1.53</td>
</tr>
<tr>
<td>Anti-dsDNA+</td>
<td>32 (60.4)</td>
<td>35.75±39.1 (0.003)</td>
<td>7.95±3.77 (&lt;0.0001)</td>
</tr>
<tr>
<td>Leukopenia</td>
<td>33 (62.3)</td>
<td>40.31±41.16 (&lt;0.0001)</td>
<td>8.0±3.8 (&lt;0.0001)</td>
</tr>
<tr>
<td>NPSLE</td>
<td>13 (24.5)</td>
<td>55.92±48.22 (&lt;0.0001)</td>
<td>9.08±4.95 (&lt;0.0001)</td>
</tr>
<tr>
<td>Reduced complement levels</td>
<td>36 (67.9)</td>
<td>43.82±42.45 (&lt;0.0001)</td>
<td>8.57±3.73 (0.004)</td>
</tr>
<tr>
<td>Arthritis</td>
<td>21 (39.6)</td>
<td>53.43±46.12 (&lt;0.0001)</td>
<td>8.41±4.42 (0.015)</td>
</tr>
<tr>
<td>Malar rash</td>
<td>33 (62.3)</td>
<td>47.27±42.70 (&lt;0.0001)</td>
<td>8.94±3.65 (0.002)</td>
</tr>
<tr>
<td>Renal involvement</td>
<td>27 (50.9)</td>
<td>53.41±43.71 (&lt;0.0001)</td>
<td>10.06±3.06 (0.003)</td>
</tr>
<tr>
<td>Lupus nephritis</td>
<td>11 (20.8)</td>
<td>56.87±47.68 (0.006)</td>
<td>8.1±4.66 (&lt;0.0001)</td>
</tr>
</tbody>
</table>

*Values are given as number (%) of patients. SLEDAI, systemic lupus erythematosus disease activity index; Anti-dsDNA, anti-double-stranded DNA; NPSLE, neuropsychiatric systemic lupus erythematosus; IFN-γ, interferon gamma; IDO-1, indoleamine 2, 3-dioxygenase-1; IDO-2, indoleamine 2, 3-dioxygenase-2. Data are presented as means ± SD. Significant differences are evaluated using independent samples t-test or one-way ANOVA followed by bonferroni post hoc test while studying multiple comparisons. Correlation analyses were performed using two-tailed Spearman’s rank correlation. Positive r-values represent a positive correlation. Negative r-values represent a negative correlation. p-Values lower than 0.05 are considered statistically significant.
correlation between IDO-2 mRNA expression level and SLEDAI score (Figure 2E) of SLE patients ($r = 0.727$, $p < 0.0001$). Significant differences are evaluated using independent samples t-test or one-way ANOVA followed by bonferroni post hoc test while studying multiple comparisons. p-Values lower than 0.05 are considered statistically significant.

**The segregation value of IFN-γ, IDO-1 and IDO-2 in the activity of SLE**

The diagnostic utility of IFN-γ, IDO-1 and IDO-2 to segregate the SLE patients with low and high disease activity was assessed using ROC curves. In the ROC analysis of IFN-γ area under the curve for IFN-γ plasma levels was 0.9786 (95% CI, 0.9481–1.009; $p < 0.0001$). Setting the optimal cut-off value at 16.03 gives a sensitivity of 96.15% and a specificity of 92.59% on IFN-γ ROC curve. p-Values lower than 0.05 are considered statistically significant (Figure 3C).

**Discussion**

SLE is a multifactorial autoimmune disease which is diagnosed by several clinical and laboratory clues [1]. In addition to the complexity associated with the diagnosis of SLE, lack of a reproducible laboratory test to characterize the disease activity is a major issue. A reliable biomarker could be helpful in predicting prognosis and determining therapeutic approaches for clinicians.

IDO is a rate-limiting enzyme in kynurenine pathway which is mostly known for its immunosuppressive properties over T-cell responses and promotion of peripheral tolerance, especially in autoimmunity and cancer [9, 19]. Although different mechanisms including tryptophan depletion and increasing proapoptotic kynurenines have been proposed, the detailed mechanism remains unrevealed. Enhanced IDO activity has been reported in different autoimmune disorders including SLE [14]. Winder
et al. [20] reported that increased tryptophan degradation and IDO activity in SLE patients could be associated with disease activity. Increased IDO expression was also reported by Pertovaara et al. [13] among SLE patients and it was shown to be elevated in those with immunologically active disease, especially during sunny seasons. However, detailed clinical analyses and monitoring of IDO activity among SLE patients are still needed which could reflect the immunosuppressive functions of IDO. IFN-γ is a Th1-dependent inflammatory cytokine [6] which is also overproduced in SLE and is correlated with the disease activity [7]. It is known as the main inducer of IDO expression among other inflammatory cytokines [10]. Although, assessment of IDO activity by means of HPLC is a more precise method, it is an expensive procedure which could not be implemented as a routine laboratory test [13, 14].

Figure 2: Correlation among IDO-1 and IDO-2 mRNA expression, IFN-γ plasma level and activity in SLE patients.

(A) Relationship between IDO-1 mRNA expression and IFN-γ level. IDO-1 positively correlated with IFN-γ (Spearman correlation coefficient \( r = 0.722, p < 0.0001 \)). (B) Relationship between IDO-2 mRNA expression and IFN-γ level. IDO-1 positively correlated with IFN-γ \( (r = 0.682, p < 0.0001) \). (C) Relationship between IFN-γ level and SLEDAI score. IFN-γ positively correlated with SLEDAI score \( (r = 0.907, p < 0.0001) \). (D) Relationship between IDO-1 mRNA expression and SLEDAI score. IDO-1 positively correlated with SLEDAI score \( (r = 0.675, p < 0.0001) \). (E) Relationship between IDO-2 expression and SLEDAI score. IDO-2 positively correlated with SLEDAI score \( (r = 0.727, p < 0.0001) \).
Therefore, evaluating the mRNA expression of IDO isoforms along with IFN-γ could precisely represent IDO activity among SLE patients.

In the present study, we found that the expression of IDO-1 and IDO-2 were noticeably elevated in the PBMCs of patients with SLE (Figure 1A and B). IDO-1 and IDO-2 are two important isoforms which are not only involved in immune modulation but also interact with each other in the T-cell response regulation [8]. Plasma levels of IFN-γ were also increased among SLE patients. Conducting correlation studies, IDO expression was found to be correlated with the IFN-γ secretion level positively (Figure 2A and B). Although SLE patients in remission did not have any ongoing IFN-γ production (Figure 1C), IDO expression was markedly increased (Figure 1A and B). This may suggest that IDO expression could be regulated by other cytokines such as IFN-α, as previously suggested [21]. More importantly, there was a significant positive correlation between IDO expression and IFN-γ plasma level with the severity of SLE (Figure 2C–E). In this case, patients with higher SLEDAI scores had significantly elevated levels of IFN-γ and IDO in comparison to those with lower SLEDAI scores. The mRNA expression of IDO-1 and IDO-2 and plasma level of IFN-γ were also analyzed regarding the demographic and clinical characteristics of SLE patients. The subsequent statistical results are summarized in Table 2.

Our study showed that IDO expression not only correlates with the onset of SLE but also with SLEDAI score. Accordingly, IDO could be introduced as a suitable determinant and potential biomarker for the disease activity along with IFN-γ. Moreover, while SLEDAI score is decreased in response to effective therapies, these markers could be used as indices to track drug responsiveness. Although this preliminary study is promising for evaluation of patients with SLE, before applying the results in the clinical setting a large-scale validation study is needed.

The underlying mechanisms why SLE patients with IDO overexpression experience higher disease activity remain elusive. Although IFN-γ is the main inducer of IDO expression, other inflammatory factors and cytokines downstream or upstream of IDO could be involved in the process [13]. Therefore, IDO overexpression may not be

Figure 3: ROC curve analysis of IDO-1 and IDO-2 mRNA expression and IFN-γ plasma levels to distinguish SLE patients with low disease activity from high.

(A) Area under the curve for IFN-γ plasma levels was 0.9786 (95% CI, 0.9481–1.009; p < 0.0001). (B) Area under the curve for IDO-1 mRNA expression was 0.7229 (95% CI, 0.5848–0.8610; p = 0.005382). (C) Area under the curve for IDO-2 mRNA expression was 0.7001 (95% CI, 0.5539–0.8464; p = 0.01246).
able to compensate the inflammation initiated in SLE. Further investigations regarding the network of pathways and cytokines collaborating with IDO are needed. However, it is obvious that IFN-γ-mediated IDO expression is insufficient to control SLE severity and it is elevated upon SLEDAI score increase.

In conclusion, IDO in two major isoforms is overexpressed among SLE patients compared to healthy controls. Moreover, expression of IDO and IFN-γ are associated with the severity of SLE and could be used as helpful biomarkers for the determination of disease severity and track the effectiveness of therapies in SLE. However, IFN-γ is a more reliable determinant regarding the area under the ROC curve, sensitivity and specificity. Altogether, simultaneous detection of IDO and IFN-γ could provide clinicians with beneficial diagnostic information which could be employed during treatment.

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