Comparison of Cytokine Expression in Multiple Sclerosis Patients and Healthy Volunteers

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Abstract- Multiple sclerosis (MS) is an autoimmune disease with the impaired balance of CD4+T cells. This trial is a descriptive study to evaluate the expression of CD4+T cell cytokines, interleukin (IL) -2, IL-4, IL-17, TGF-β, and respectively related transcription factors, including T-bet, GATA3, RORγt and FoxP3 in MS patients. Sixteen relapsing-remitting MS (RRMS) patients receiving interferon beta (IFN-β)1a in the stable phase of the disease and 14 healthy control volunteers (HCs) were enrolled in this study. The expression of cytokines and transcription factors was evaluated in peripheral blood mononuclear cells (PBMCs) of patients using real time PCR. The results of this study showed that the expression of IL-2 (P≤0.05), IL-4 (P≤0.05), IL-17 (P≤0.05) and RORγt (P≤0.01) in PBMCs of RRMS patients were significantly higher than those in HCs. The expression of TGF-β, GATA3, and FoxP3 were higher but the RORγt expression was lower in the patients than HCs without reaching significant value. Observed results indicated differences in immune system cytokines of healthy volunteers and the patients that were in the stable phase and under immunomodulatory therapy especially in proinflammatory mediators. Therefore, any therapeutic strategy to restore the immune system balance is desirable in RRMS patients.

Keywords: Multiple sclerosis; T cells; Cytokine; Transcription factor

Introduction

Multiple sclerosis (MS) is an inflammatory disease that usually occurs in young people. A disabling disease that affects the brain and spinal cord and more than two million people are affected worldwide (1). Although MS etiology is not fully understood, antigen-activated T cells play an important role. Neural demyelination in MS happens after the activation of peripheral autoreactive T cells. Antigen-activated naïve CD4+ T cells differentiate into the various lineage of effector T cells like T helper (Th)1 and Th2. In MS disease, IFNγ-producing Th1 cells were thought originally to be the main pathogenic CD4+ T cells (2). T helper 2 cells produce Type 2 cytokines, including interleukin (IL)-4, IL-5 and IL-13, mediate protective immunity to extracellular pathogens, and have protective role in autoimmune diseases. Therefore maintenance of immunity balance needs proper regulation of the Th1/Th2 balance (3).

Naïve T cells can differentiate into proinflammatory Th17 or tissue protective inducible T regulatory (Treg) cells depending on the expression of RAR-related orphan receptor γ (RORγt) or forkhead box P3 (FoxP3) as transcription factors (2). The discovery of Th17 cells that secret IL-17, a proinflammatory mediator, and the critical role of Th17/Treg balance in MS pathogenesis provided a better understanding of MS pathogenesis. Treg cells downregulate the immune system and secrete anti-inflammatory cytokines like transforming growth factor β (TGF-β) (4). The first step of the creation of MS disease

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Cytokine expression in MS patients

is activation of peripheral Th1 and Th17 followed by cellular migration of activated lymphocytes across the blood-brain barrier. This process follows by local secretion of proinflammatory cytokines and chemokines that lead to the subsequent destruction of myelin and axonal loss (5). Several studies stated that the development and progression of MS in humans and experimental autoimmune encephalomyelitis (EAE) in rodents is due to the imbalance of proinflammatory T cell responses, such as Th1 and Th17, and anti-inflammatory T cell responses, such as Th2 and Treg (6-8). IL-2 producing Th1 cells and IL-4 producing Th2 cells utilize T-box expressed in T cells (T-bet) and GATA-binding protein 3 (GATA3) as lineage regulators, respectively (5).

Currently prescribed treatments for alleviating disease progression use immunomodulatory drugs such as recombinant interferon beta (IFN-β)-1a seems to influence this pathogenic process by reduction of T cell activation and trans-migration as well as cytokines secretion (9). However, this drug family only works as first-line of treatment, and the patient responses are variable because of immunopathologic heterogeneity (10). Accordingly, in the present study the expression level of cytokines and transcription factors related to Th1, Th2, Th17, and Treg cells were assessed in PBMCs of Relapsing Remitting MS (RRMS) patients receiving IFN-β-1a and compared with those in healthy volunteers.

Materials and Methods

Patients and healthy volunteers

This study was designed as a cross-sectional study. All patients were recruited to this study, referred by neurologists. The patients had definite MS according to McDonald's criteria (11) and were under treatment by IFN-β-1a (ReciGen, CinnaGen company, Iran) therapy by the time of blood collection. They were classified as RR according to standard disease course neurological disability criteria (12) and was evaluated by the expanded disability status scale (EDSS) (13). Patients were included in the study according to the following criteria: (i) EDSS score between zero and five without attack; (ii) age between 20 and 45 years; (iii) receiving 30 µg I.M. of IFN-β-1a weekly at least for one year. Patients who treated with immunosuppressive drugs and multivitamins and/or suffered from any other autoimmune diseases such as lupus erythematosus, rheumatoid arthritis, and etc., and/or with other conditions that may affect the immune system such as pregnancy, malnutrition (body mass index <18.5) and overweight, were not enrolled in the study. Patients who change their treatment protocol were excluded. Healthy control subjects had no history of MS or another autoimmune disease. Sixteen patients (12 female and 4 male) and 14 HCs (8 females and 6 males) fulfilled the inclusion criteria and participated in this study.

Cell isolation, RNA extraction and real-time polymerase chain reaction (PCR) analysis

Venous blood samples were collected from all participants into 6 ml EDTA-coated vacutainer tube. Fresh blood was centrifuged at 150×g for 5 minutes, and the supernatant was removed. As previously described (14), PBMCs were isolated by density gradient centrifugation with Ficoll-Histopaque solution (Biosera, UK). Total RNAs was extracted from fresh PBMCs using Viogene extraction kit (Viogene, Inc., Taipei, Taiwan), according to manufacturer’s instruction. The concentration and purity of extracted RNAs were confirmed with a NanoDrop spectrophotometer (NanoDrop Technologies, USA). Total RNAs were reverse-transcribed to cDNA using cDNA synthesis kit (Takara Bio Inc., Japan). Standard quantitative real-time PCR was carried out by ABI Step One System (Applied Biosystems Foster City, CA, USA) using SYBR Green PCR Master Mix reagents (Applied Biosystems Foster City, CA, USA). The specific primers of target genes, IL-2, IL-4, IL-17, TGF-β, T-bet, GATA3, RORγt, and FoxP3 were designed by Primer Express 3 software (Table 1). Real-time PCR reactions were performed using 100 ng cDNA samples, 1XQuantiTech primers, and 1XQuantiTech SYBR-Green PCR master mix in a total volume of 20 µl. The PCR was run at 94°C for 15s, 55°C for the 20s and 72°C for 20s in 40 cycles. PCR efficiency was determined using LinReg PCR software. Expression levels of genes were normalized by β-actin as housekeeping reference gene and calculated by the 2ΔCt method:

\[ ΔCt = Ct \beta-actin−Ct \text{ target gene (15).} \]

Statistics

Statistical analysis was performed using SPSS 18.0 package (SPSS Inc, Chicago, IL). Data are expressed as mean±S.D. The normality distribution of values was tested using the Kolmogorov–Smirnov distribution test. The independent sample test was used to assess the significance of differences. If data had normal distribution, Independent sample T-test and if they did not have a normal distribution, a nonparametric Mann–Whitney U test was used. Statistical significance of the correlation between genes was performed by Pearson's
correlation test. Results were considered statistically significant when $P \leq 0.05$.

### Table 1. Sequence and information of primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2</td>
<td>F: GCCAAAATCCACCGAGGATGCTCA</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>R: ACGTTGATTGTCGTAATATTAGTCCCT</td>
<td>26</td>
</tr>
<tr>
<td>IL-4</td>
<td>F: GCACTGTAAGCAGGAAGAAC</td>
<td>22</td>
</tr>
<tr>
<td>IL-17</td>
<td>F: GGGGCTGGCTCTGTCGTA</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>R: AAGTTCGTTGCTGCCCACATCA</td>
<td>20</td>
</tr>
<tr>
<td>TGF-β</td>
<td>F: CTCTCGAATGCGCCACAGA</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>R: AACCTAGATTGCGGCGATCT</td>
<td>20</td>
</tr>
<tr>
<td>RORγt</td>
<td>F: GAAATGTTGTCGTTAGATGTCG</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>R: GCCACCCTTATGCTCCTTACA</td>
<td>20</td>
</tr>
<tr>
<td>FoxP3</td>
<td>F: GCCAAAGTTGTTTGTGACGTGACA</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>R: AGGCTGGTTGAAATGGGACTGA</td>
<td>21</td>
</tr>
<tr>
<td>GATA3</td>
<td>F: AGATGGCAGGGGACACTACT</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>R: CTTGCCTGGGCTGTTAAGA</td>
<td>20</td>
</tr>
<tr>
<td>T-bet</td>
<td>F: TGCTGACGGATCCATAAGTAC</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>R: TCTGCCCTGGCCGGATAC</td>
<td>20</td>
</tr>
<tr>
<td>β-actin</td>
<td>F: CCTGCGACCCACACATAAT</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>R: GCCGATCCACACGAGTACT</td>
<td>20</td>
</tr>
</tbody>
</table>

F: forward, R: reverse

### Results

#### General information

A total of 16 RRMS volunteer patients (73% female and 27% male) and 14 volunteer HCs (57.1% female and 42.8% male) participated in this study. The mean age of RRMS patients and HCs were 39.7±3.8 and 36.3±4.2 years, respectively. In RRMS patients, the mean disease duration, mean relapse rate (relapses/year), and mean EDSS score was 8±0.8 years, 2.4±0.3 and 2.3±0.8, respectively.

#### Gene expression of IL-2 and T-bet in isolated PBMCs

The results of quantitative measurement of IL-2 expression in freshly isolated PBMCs of RRMS and HCs subjects showed that patients expressed significantly higher level of IL-2 when compared to the HCs ($P=0.05$). To the contrary, T-bet expression level was lower in RRMS patient vs HCs. However the difference was not significant ($P=0.07$) (Table 2, Figure 1). Statistical analysis showed no significant correlation between IL-2 and T-bet in both groups of study.

### Table 2. ΔCt values and fold change of genes obtained from PBMCs of RRMS patients and HCs

<table>
<thead>
<tr>
<th>ΔCt of gene expression in PBMCs</th>
<th>Genes</th>
<th>RRMS Patients (N=16)</th>
<th>HCs (N=14)</th>
<th>Fold change</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2</td>
<td>-15.9±0.9</td>
<td>-16.5±0.7</td>
<td>1.70</td>
<td>0.050 $^d$</td>
<td></td>
</tr>
<tr>
<td>IL-4</td>
<td>-8.6±0.9</td>
<td>-9.4±1.1</td>
<td>1.68</td>
<td>0.009 $^d$</td>
<td></td>
</tr>
<tr>
<td>IL-17</td>
<td>-7.8±0.9</td>
<td>-8.6±1.2</td>
<td>1.52</td>
<td>0.025 $^d$</td>
<td></td>
</tr>
<tr>
<td>TGF-β</td>
<td>-4.6±0.9</td>
<td>-4.9±1.4</td>
<td>1.34</td>
<td>0.452</td>
<td></td>
</tr>
<tr>
<td>T-bet</td>
<td>-7.1±0.8</td>
<td>-7.1±0.7</td>
<td>0.70</td>
<td>0.074</td>
<td></td>
</tr>
<tr>
<td>GATA3</td>
<td>-7.5±0.7</td>
<td>-7.7±0.9</td>
<td>1.14</td>
<td>0.353</td>
<td></td>
</tr>
<tr>
<td>RORγt</td>
<td>-7.0±0.7</td>
<td>-8.1±1.2</td>
<td>1.52</td>
<td>0.008 $^d$</td>
<td></td>
</tr>
<tr>
<td>FoxP3</td>
<td>-9.3±1.0</td>
<td>-9.9±1.4</td>
<td>1.32</td>
<td>0.158</td>
<td></td>
</tr>
</tbody>
</table>

a Data are reported as mean ±S.D.

b $ΔCt = Ct$ β-actin − $Ct$ target gene
c Gene expression of RRMS patients with HCs

d $P<0.05$

#### Gene expression of IL-4 and GATA3 in isolated PBMCs

The results showed that IL-4 expression level was significantly higher in PBMCs of RRMS patients when compared to the HCs ($P<0.01$). The PBMCs expression level of GATA3 showed no significant difference in RRMS patients and HCs (Table 2, Figure 1). Statistical analysis showed a high correlation coefficient for IL-4 and GATA3 pair of genes ($r = 0.88^{++}$ for RRMS and $r = 0.83^{++}$ for HCs), and it was obtained significant ($P<10^{-3}$ in both RRMS and HCs).
Gene expression of IL-17 and RORγt in isolated PBMCs
The expression of IL-17 was obtained higher in PBMCs of RRMS patients when compared to HCs at a significant level (P<0.05). The results of the RORγt expression was higher in PBMCs of patients compared to HCs (P<0.01) (Table 2, Figure 1). The correlation coefficient analysis of IL-17 and RORγt was statistically significant (P<10^{-3} in both RRMS and HCs) (r=0.77** for RRMS and r=0.94** for HCs).

Gene expression of TGF-β and FoxP3 in isolated PBMCs
The expression of TGF-β, as one of the major Treg cell cytokines, was evaluated and the results showed a non-significant higher level in RRMS patients versus HCs subjects. Similarly, FoxP3 expression level showed the non-significant difference in RRMS patients and HCs (Table 2, Figure 1). There was no significant correlation between TGF-β and FoxP3 pair of genes in RRMS patients and HCs.

Discussion
MS is a chronic disease with central nervous system demyelination; the main pathogenic factors that cause this disease are excessive activation of peripheral autoreactive T cells, disturbing the balance of Th1/Th2, and higher activity of Th17 cells (1, 16). The prevalence of MS in many countries is growing fast, and the number of individuals who are newly diagnosed MS has been increased manifold (17). Some immunomodulatory and anti-inflammatory drugs that are generally prescribed for the treatment of MS cannot fully repair the imbalance of
the immune system and inhibit the progression of the disease (9). In this descriptive study, the expression of one cytokine and related transcription factor of Th1, Th2, Th17 and Treg cells in freshly isolated PBMCs of RRMS patients and healthy volunteers were evaluated. Results showed a significantly higher expression of IL-2, IL-4, IL-17, and RORγt in studied RRMS patients in comparison to studied healthy volunteers.

Based on a previous study on cerebrospinal fluid infiltrating immune cells in animal models, MS is considered as a CD4+ Th1 mediated autoimmune disease (18). Main cytokines of these cells including IFN-γ, TNF-α, and IL-2 believed to initiate MS disease and also to help oligodendrocyte damage progression and previously considered as a marker of relapses (19). The higher expression level of IL-2 in RRMS patients, observed in the present study (Table 2, Figure 1), may admit differences in Th1 cells even in the stable phase of disease that may play a critical role in the upcoming relapses. Other studies showed upregulation of IL-2 and IFN-γ during exacerbated condition (20, 21). Honarvar et al. (22) showed the expression of IFN-γ and T-bet increased (not significant) in RRMS patients receiving IFN-β-1a during 6 months. In addition, IFNγ was significantly higher in newly diagnosed and RRMS patients, also in remission phase of patients receiving immunomodulatory drugs, and in secondary progressive MS patients in comparison to healthy people (23).

It has been shown that another subset of CD4+ T cells called Th17, plays an important role in host defense (24). Abnormally increased activity or number of Th17 cells results in autoimmune diseases such as MS and also increased expression of IL-17 has been shown in PBMCs and lesions of MS patients (25, 26). Migration of Th1 and/or Th17 into the brain of EAE mice leading to myelin damage and the formation of plaques at distinct sites that causes functional loss (2). Significant higher expression of IL-17 and RORγt and clear correlation between this pair of genes that were observed in present study in RRMS patients confirm the hazardous role of Th17 cells in this disease. Assessment of IL-17 in different phases of disease revealed that during the onset of MS, IL-17 production in PBMCs was significantly higher than healthy subjects, however, in remission phase of RRMS patients, it was almost the same (23). Observed results of this study showed that even in remission phase, IL-17 might be higher in patients in comparison to HCs (Table 2, Figure 1). Th17 cells, in addition to Th1 cells, may involve in the progression of diseases even when patients are treated with immunomodulatory drugs. Two related studies showed RRMS patients under IFN-β-1a therapy had higher level of IL-17 (P=0.07) and lowered FoxP3 (P=0.008) after 6 months survey (27,28). The aims of new therapies for MS are reducing the number of relapses and slowing the progression of disability. However, this treatment is not always completely effective (10,29). In one-third of the RRMS patients, who received these treatments, recurring attacks and/or disability were increased. The reason may be that the main function of these immunomodulatory drugs is to reduce the activity of Th1, but not Th17 cells, as Axtell et al., (30) demonstrated. In confirmation of these results, we found that the level of T-bet expression was lower in RRMS patients than HCs. As expected for the effects of IFN-β-1a, the level of IL-4 and GATA3, the Th2 related cytokine and transcription factors, was higher in patients who received this drug. Correlation analysis showed a positive and significant relationship between these two genes.

Expression of T cell transcription factors is controlled by some cytokines. For example, expression of IL-17 and RORγt in Th17 depends on the presence of pro-inflammatory cytokines such as IL-6 and TGF (31). TGF-β producing Treg cells reduce the disease progression and recurrent relapses (32). In the present study, the expression of TGF-β was non-significantly higher in HCs than patients and the expression of FoxP3 showed no difference between patients and HC group. Prior studies showed that the activity of Treg cells and the expression of FoxP3 in PBMCs of RRMS patients decreases (33), also higher percentage of Treg cells in autoimmune disease does not mean that they are able to control the immune response (4). Equal values of FoxP3 and TGF in this study could be due to the effect of IFN-β-1a (Table 2 Figure1). Due to the high variability of values and low number of studied patients in this study further studies are needed to determine the immunological difference between patient and healthy individuals and to investigate the cellular and molecular effects of different medications on MS patients in different phases of the disease.

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