ORIGINAL ARTICLE
Iran J Allergy Asthma Immunol
April 2015; 14(2):208-216.

Cytokine Gene Expression in Newly Diagnosed Multiple Sclerosis Patients

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Received: 9 February 2014; Received in revised form: 31 May 2014; Accepted: 21 July 2014

ABSTRACT

Multiple Sclerosis (MS) is characterized by multiple areas of inflammation, demyelination and neurodegeneration. Infiltrating Th1 CD4+ T cells secrete proinflammatory cytokines. They stimulate the release of some cytokines, expression of adhesion molecules and these cytokines may cause damage to the myelin sheath and axons.

In this study, we analyzed plasma levels and gene expressions of five important cytokines in the new diagnosed MS Patients by ELISA and Real time PCR. PCR amplifications were performed to determine the IL-17, IL-23, IL-10, IL-27 and TGF-β mRNA expression levels using the SYBR Green PCR Kit.

Our results showed significant decrease in IL-10, IL-27 and TGF-β but there was no significant difference in the IL-17 and IL-23 between patients and healthy controls. Altogether, our results indicated that dysregulation of cytokines, mainly increased expression of pro-inflammatory cytokines and decreased expression of inhibitory cytokines occurred in MS patients.

This study may shed light to the probable role of these cytokines in neurodegeneration mechanism and current or future use of cytokines in managing and treatment of multiple sclerosis.

Keywords: Autoimmunity; Cytokines; Inflammation; Multiple Sclerosis; Neurodegeneration

INTRODUCTION

Multiple Sclerosis (MS), a chronic inflammatory (CNS), is the most common cause of neurological disability mainly affecting young adults.[1] The incidence and prevalence of MS in Iran has been

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Published by Tehran University of Medical Sciences (http://ijaai.tums.ac.ir)

growing without explanation, especially in females.[2] It is generally believed that MS is an autoimmune disorder.[3-5]

In addition to T helper-1 (Th1)-lymphocytes, Th17-cells that secrete interleukin (IL)-17 are believed to be important in the pathogenesis of MS.[6] A study has demonstrated that the IL-23/Th17 pathway plays a role in EAE.[7] The important role of IL-23 in the expansion of CNS inflammation was a reflection to be due to its role in promoting IL-17 production, but recently it has been found that IL-23 has an important role in homing to the CNS and survival of myelin-specific T lymphocytes in the CNS microenvironment.[8] IL-23 may affect T cell trafficking by making changes in chemokine receptors, such as chemokine receptor 6 (CCR6) that are highly expressed on human Th17 lymphocytes.[9,10] IL-23 driven Th17 lymphocytes also cause inflammation of the optic nerve and create lesions similar to Neuromyelitis Optica.[11] These data suggest that the effector T lymphocyte cytokines can play important role in the migration of the T cells to specific regions within the CNS. Unusual recruitment of these immune cells cause functional losses.[12]

The essential phase of inflammation in the autoimmune disorders is altered balances between pro-inflammatory versus anti-inflammatory cytokines in the disease process. MBP-specific T lymphocytes isolated from MS patients have shown that T lymphocytes have an essential role in the MS pathology.[13,14] High levels of Th1 cytokines are mostly obvious during EAE/MS relapse, but there are high levels of Th2 cytokines during remission in MS patients.[15] Altered reactivity of PBMC and their cytokines may influence the clinical course of MS and also altered reactivity of immune system cells modulate this disease.[16]

Symptoms worsen in relapsing/remitting MS patients after IFN-γ administration. This is also shown in other Th1-related disorders, whereas it is not shown in Th2 type disorders.[17,18]

Mice with deficiency in IL-12α (IL-12p35, part of the Th1 prototype), are susceptible to EAE. In the same way, IL-12R β2-deficient mice expand severe clinical manifestations of EAE, whereas mice with deficiency in IL-12p40 are resistant to EAE.[19-21] These paradoxical data show that an imbalance in the Th1/Th2 cells cannot elucidate the overall immunopathogenic mechanisms of MS. IL-23, not IL-12, is crucial for induction of EAE.[22] Moreover, Th17 cells are expanded by IL-23, and can induce EAE when transferred into naive mice adoptively.[23,24] These Th17 lymphocytes were significantly reduced in the CNS of mice with IL-23p19-deficiency. These results suggested that Th17 lymphocytes exacerbate autoimmunity.[25,26]

The MS cytokine profile was more clarified after Th17 discovery[10] and one study showed that the levels of IL-17 produced by MBP-stimulated peripheral blood cells was associated with the lesions activity in MS patients.[27]

TGF-β is a major cytokine in the production of regulatory T lymphocytes (Tregs). These cells inhibit the autoimmune responses and protect CNS against inflammatory damages. Tregs play important role in the regulation of Th1/Th2 and Th17 lymphocytes. As a result, the production of pathogenic Th17 cells provokes autoimmunity, but the generation of Tregs inhibits autoimmune tissue damage.[28] On the other hand, IL-27 is an inhibitor of Th17 lymphocyte differentiation and prevent inflammatory demyelination of EAE in animal models.[29] Some studies showed that IL-12, IL-23 and IL-27 are associated to the group which stimulates Th1 type of immune response.[30]

Jager et al. studies have shown that Th1, Th17, and Th9 lymphocyte, but not Th2 cells, induce EAE. In addition, each T-cell subset induces disease in an individual pathological approach. The pathological variation in MS lesions may result in different effects of reactive T lymphocytes against myelin.[31,32] Treatment with IFN-β can decrease IL-23 mRNA levels[33] and suppress human Th17 lymphocyte subset differentiation, hence the Th17 may be an additional target of IFN-β therapy.[34] IFN-β plays an important role in inhibiting Th17 cells by its effect on innate immune system cells and stimulates IL-27 synthesis[35] and it may also induces IL-10 production which act as a negative regulatory cytokine.[33]

The failure of IFN-β therapy in some relapsing/remitting MS patients can be related to high levels of IL-17F cytokine in these patients.[36] This cytokine suppresses Th17 expansion in MS, and may have an effect on the clinical symptoms in patients.[37] Consequently, the result of most related studies endorsed very important role of cytokine profile in disease onset and its clinical progress.

The aim of the present study was to evaluate plasma levels of these cytokines in MS patients in comparison with healthy controls to find probable association between cytokine profile and MS disease.

MATERIALS AND METHODS

This study was approved by the Ethical Committee on Human Research, Shahre Kord University of Medical Sciences. The volunteers were referring to MS Clinic of Isfahan Kashani Hospital. They were informed about the procedure of the study and those who were willing to participate, donated blood sample and signed an informed consent.

Study Groups

45 newly-diagnosed MS patients [33 females and 12 males, (39 RRMS, 1 PPMS and 5 SPMS)] and 45 age- and gender-matched healthy control (HC) individuals were recruited in the study. New cases were those who had at least one episode of focal neurological signs, symptoms such as Fatigue and numbness, weakness in a leg or legs, visual problems and positive Magnetic resonance imaging MRI findings (according to "Revised Mc Donald criteria" and exclusion of other possible differential diagnosis (clinically and para clinically). All samples were taken during relapse but before taking any drugs including "Methyl prednisolone, Beta-interferone,) or immuno suppressives and immuno modulators. The members of healthy control group were selected from Blood Bank organization donors. The donors' health was confirmed by Physician of this Organization. Control group members did not have any drug administration. All blood samples were collected during relapse phase before any drug administration. Patients were clinically diagnosed with MS according to the McDonald criteria with EDSS between 0-3.[38]

Isolation of Plasma and PBMC from Whole Blood

Blood samples were obtained from patients and healthy volunteers and transported on ice to the laboratory. Plasma were removed and kept frozen at −80°C until cytokine determination. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Hypaque density gradient (Lymphodex, Germany). PBMCs were washed three times with Phosphate buffered saline (PBS) and the pellet was resuspended in a RNA protect cell solution (Qiagen, Germany) and stored at -80°C until use for RNA extractions.

RNA Extraction and cDNA Synthesis

Total RNA was isolated from cell lysate of 10^6 PBMC using miRCURY RNA Isolation Kit (Exiqon Denmark) under RNase-free condition according to the manufacturer's protocol. The quality and quantity of RNAs were checked using agarose gel electrophoresis and spectrophotometric analysis. DNase treated RNA was reverse transcribed using RevertAid First Standard synthesis kit (Fermentas, Lithuania) according to the manufacturer's instruction. The samples were then reverse transcribed using 0.5 µg of RNA under conditions of 65 °C for 5 min, 25 °C for 5 min, 42 °C for 60 min and 70 °C for 5 min.

Real-Time PCR

PCR amplifications were performed to determine the IL-17, IL-23, IL-10, IL-27 and TGF-β mRNA expression levels using the SYBR Green PCR Kit (Fermentas, Lithuania) in a total volume of 25 µl, containing 1.5 µl cDNA samples, 5 picomoles of each primer and 12.5 µl of SYBR Green PCR Master Mix. The PCR primers that were used for real-time PCR are shown in Table1. Real-time reverse transcription PCR (RT-PCR) was run on the ABI Step one Plus (Applied Biosystems USA). The PCR conditions were as follows: initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 seconds and 60°C for 60 seconds. Melting curve analysis of the PCR product was performed after amplification to identify all PCR products. Real time PCR reactions were performed in triplicate. The Ct values for each gene were normalized to endogenous control gene, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and calculation was performed using $2^{-\Delta ct}$ method.

Cytokine Analysis

Plasma levels of MS-relevant cytokines (IL-10, IL-17, IL-23, IL-27, and TGF-β) were determined by ELISA system using ELISA Reader (Hyperion, USA)

IL-17A, IL-10 and TGF-β were assessed using commercial ELISA kits from Bender Med Systems (Vienna, Austria), and IL-23 and IL-27 cytokines were assessed using Abcam company kits (Abcam, UK) according to the manufacturer's instructions.

The absorbance of each well was read at 450nm and the concentrations were determined using standard curves. Cytokine levels were expressed as picogram/ml (pg/ml).

Statistical Analysis

Statistical analysis was performed using SPSS

version 16. Student's independent t-test was used to determine the significant difference; $P<0.05$ was considered as significant.

RESULTS

The basic information of the 45 recruited patients (M=12, F=33) and controls (M=12, F= 33) is summarized in Table2. There was no significant difference in the mean age of case and control groups.

Real Time PCR IL-17, IL-10, IL-23, IL-27 and TGF-β Gene Expression

The mRNA levels of IL-17, IL-10, IL-23, IL-27, TGF-β and GAPDH as a housekeeping gene were quantified by quantitative Real-time PCR. The mRNA mean expressions of IL-17, IL-10, IL-27, TGF-β were different in patient and control groups, which displayed a significant increase in IL-17 and decrease in IL-10, IL-27 and TGF-β in patients group ($p=0.015$, $p=0.005$, $p=0.027$, $p<0.001$). The mRNA mean expressions in IL-23 was not significantly different in the two groups, however, it was higher in patients group ($p=0.079$) (Table 3 and Figure1).

ELISA Evaluation Result

In the present study plasma levels of IL-17, IL-10, IL-23, IL-27, and TGF-β were evaluated in patients and controls. The results showed a considerable increase in the plasma levels of IL-17A in MS patients compared with healthy controls, however, this difference was not significant ($p=0.065$).The plasma levels of IL-23 was higher in patients group, however, it was not significant ($p=0.078$).The plasma levels of IL-10, IL-27 and TGF-β in MS patients were significantly lower than healthy controls ($p<0.001$, $p<0.001$, and $p=0.013$) (Table 3 and Figure 2).

Table1. Primers used in real-time polymerase chain reaction.

Target gene		Primers	Product size
Human IL-17 A	Forward:	5'- CTGTCCCCATCCAGCAAGAG -3'	132
	Reverse:	5′- AGGCCACATGGTGGACAATC -3'	
Human IL-10	Forward:	5'- GCCGTGGAGCAGGTGAAG-3'	144
	Reverse:	5′- AGTCGCCACCCTGATGTCT-3'	
Human IL-23p19	Forward:	5'-GTGGGACACATGGATCTAAGAGAAG-3'	124
	Reverse:	5′- TTTGCAAGCAGAACTGACTGTTG-3'	
Human IL-27	Forward:	5'- CCTGGTTCAAGCTGGTGTCT -3'	167
	Reverse:	5'- CTCCTGGCAGGTGAGATTCC -3'	
Human TGF-β	Forward:	5'- CAGCAACAATTCCTGGCGATA-3'	136
	Reverse:	5′- AAGGCGAAAGCCCTCAATTT-3'	
Human GAPDH	Forward:	5'-CCACTCCTCCACCTTTGACG -3'	107
	Reverse:	5′- CCACCACCCTGTTGCTGTAG-3'	

Table2. Demographic characteristics of MS patients and healthy Controls studied.

Variables	Groups		*P* value
	MS (n=45)	Control (n=45)	
Age (year)	32.15±10.69	33.28±9.92	0.60
Gender (Female/male)	33/12	33/12	1
Age range	17-58	17-58	1

Data are Mean ± SD *P* values derived from T-test

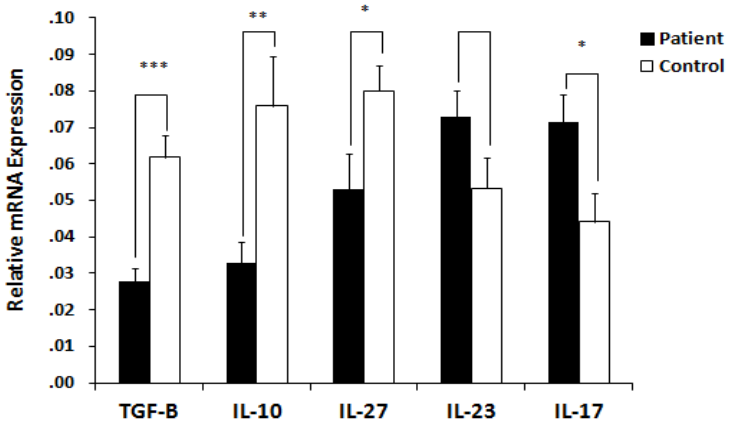

Figure 1. mRNA expression of TGF-β, IL-10, IL-27, IL-23, and IL-17 from PBMC in MS patients and healthy controls. Data are expressed as the mean ± standard deviation of the mean.

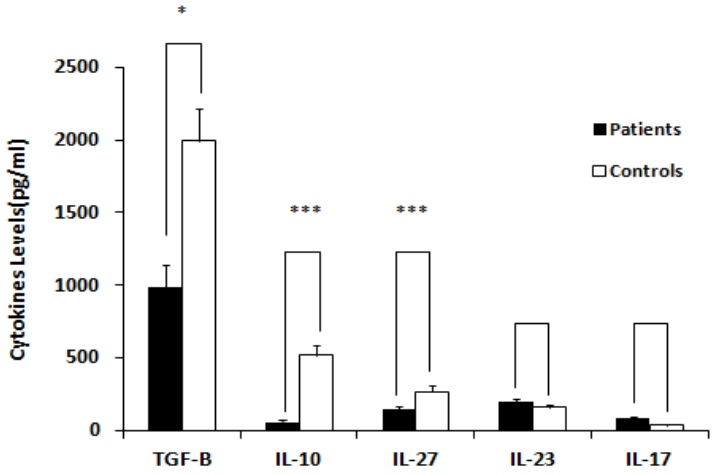

Figure 2. Plasma cytokine levels in MS patients and healthy controls. Data are expressed as the mean ± standard deviation of the mean.

Table 3. Comparison of cytokine levels and relative mRNA expression in MS patients and healthy controls

Variables	MS (n=45)	Control (n=45)	*P Value*
IL-17A Cytokine Level	81.91±15	35.24±10	0.065
Il-17 Relative mRNA Expression	0.071354±0.0200	0.044097±0.0250	0.015
IL-10 Cytokine Level	56.13±23	519.66±65	<0.001
Il-10 Relative mRNA Expression	0.0329±0.0100	0.0759±0.0267	0.005
IL-23 Cytokine Level	194.089±26	158.51±17	0.078
Il-23 Relative mRNA Expression	0.0729±0.0100	0.0533±0.0200	0.079
IL-27 Cytokine Level	144.86±27	266.48±45	<0.001
Il-27 Relative mRNA Expression	0.0531±0.0287	0.0800±0.0254	0.027
TGF-β Cytokine Level	989.68±155	1992.4±220	0.013
TGF-β Relative mRNA Expression	0.0278±0.0150	0.0618±0.0146	<0.001

Data are Mean ± SD *P* values derived from T-test

DISCUSSION

In the present study, we found an alteration in plasma levels of Pro-inflammatory (IL-17, IL-23) and immunoregulatory cytokines (IL-10, IL-27 and TGF-β) in newly-diagnosed patients with MS.

Our study has also shown that the gene expression of these cytokines was changed in PBMC of these cases in comparison with healthy controls. Our data demonstrated gene expression of IL-17 in the patients group was significantly higher than control group and plasma level of this cytokine in the patients group was increased, however, it was not significant. This can be explained by the fact that gene expression does not necessarily result in translation into complete and functional protein, and post-translational modifications or regulatory systems can suppress or limit protein secretion. However, some studies have shown that IL-17 level of MS patients are significantly higher than controls. Matusevicius et al. also have shown that in patients with MS, a high copy number of IL-17-mRNA in mononuclear lymphocytes in the cerebrospinal fluid (CSF) was present in comparison with the peripheral blood.[39] A study in Iran demonstrated significant difference in IL-17 between patients and control groups.[40] However, Kallaur et al findings did not show significant differences in IL-17 between case and control groups.[41] This discrepancy may be a result ofthe diversity in patients. In our study, we used newly-diagnosed cases of MS before any treatment. On the other hand, negative feedback regulatory mechanisms may affect IL-17 protein expression and decreased IL-17 production. In addition, this alteration may result in a difference in regulatory factors, enhancers and inhibitor molecules due to primary expression of cytokines.[42]

The present study indicated that although gene expression and plasma level of IL-23 was not significantly different between the two groups, expressions were higher in patients group. High levels of IL-23 are associated with increasing IL-17 secretion from T cells. Similar to our result, a study by Vaknin-Dembinsky et al. showed that monocyte-derived dendritic cells in MS patients produce greater amounts of IL-23 compared with healthy controls.[43]

Another study has reported that decrease of IL-17 inhibits neutrophil recruitment and a secondary damage in CNS by matrix metalloproteases release. On the other hand, the reduction of IL-23 prevents CNS inflammation. These data also demonstrated that deficiency of IL-17 is the cause of less severe EAE but deficiency of IL-23 causes no disease.[12]

On the other hand, our result demonstrated that IL-10, IL-27 and TGF-β levels in the patients group were significantly lower than the control group. These findings demonstrate inhibitory role of these cytokines on the immune system cells. These results agree with the findings of other studies which indicated that IL-10 cytokine is decreased during relapses[44] and a number of studies make clear inhibitory effects of IL-10 in MS.[45,46]

Mayer et al. reported that TGF-β1 secretion by regulatory T lymphocytes inhibit the development of EAE.[47] In addition, exogenous TGF-β1 can prevent the development of EAE and the severity of the disease was increased by TGF-β1 neutralization. Also appearance of symptoms in MS patients has been associated with TGF-β1 levels.[48] Differentiation of Th17 lymphocytes is mediated by IL-6 and TGF-*β*.

Our results showed that gene expressions and plasma level of IL-27 were significantly lower in patients group compared to healthy controls. IL-27 has inhibitory role on production of IL-6 and TGF-*β* and suppresses Th17 cell production indirectly. On the other hand, IL-27 generally has anti-inflammatory function and may be regarded as a suppressor of autoimmunity.[49] Furthermore, in the EAE model, IL-27 is a negative regulator of Th17 lymphocyte differentiation and can inhibit inflammatory demyelization.[29]

IL-27 plays a role in expansion and differentiation of IL-10-producing regulatory T cells through promoting the expression of three important molecules: ICOS (an inducible T-cell costimulator), c-Maf (the basic leucine-zipper transcription factor Maf) and IL-21.[50]

Fitzgerald DC et al. have shown that in IL-27-deficient mice, EAE was more severe but CNS inflammatory infiltration was noticeably reduced in the IL-27 treated mice.[51] Latest studies have confirmed a pro-inflammatory role of IL-27 in both innate and adaptive immune responses in chronic diseases.[52]

Some drugs in MS patients act through cytokine alterations. It was reported that mitoxantrone treatment acts in MS patients through the development of Th2 cytokines[53] and Glatiramer acetate (GA) boosts the suppressive effects of Tregs in MS.[54]

In conclusion, our results showed that a

dysregulation of cytokines, mainly decreased expression of inhibitory cytokines and increased expression of pro-inflammatory cytokines, occurred in MS patients. Further studies with more samples should be done to explore the role of cytokines in MS patients. This information is helpful for suggesting cytokines as new candidates in diagnostic and therapeutic approaches.

ACKNOWLEDGEMENTS

The study was supported by Shahre Kord University of Medical Sciences (Grant No1263, No 1268). The authors would like to appreciate Dr Seyed Hossein Hejazi for sincere technical assistance and also Dr Nahid Akbari, Mrs Afsaneh Mohamady, Mrs Saiar and Mr Ebrahim Javadi Negad for their help with patient sampling.

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