

Altered Serum Cytokine Profiles in Relapse Phase of Relapsing-Remitting Multiple Sclerosis

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ABSTRACT

Background: Multiple sclerosis (MS) is a demyelinating disease of the central nervous system and cytokines may play a role in the development of MS lesions. **Objective:** To determine levels of different cytokines in patients with relapsing-remitting MS (RR-MS) compared to healthy controls. **Methods:** Profiles of pro-inflammatory, Th1-, Th2-, and Th17-related cytokines were compared by quantitative multiplexed ELISA-based chemiluminescent assay in 44 RR-MS and 44 healthy age- and sex-matched individuals from the same ethnicity. **Results:** Among pro-inflammatory cytokines, the levels of IL-6 ($p=0.003$), IL-8 ($p=0.05$) and TNF- α ($p=0.002$) were higher in patients than controls, though IL-4 and IL-10 as well as Σ Th2 cytokines were lower in patients ($p=0.05$, $p=0.02$ and $p=0.05$, respectively). After gender classification, the higher levels of IL-4 in male patients remained significant and IL-13 also showed significantly higher levels in male patients compared to male controls ($p=0.003$ and $p=0.05$, respectively). A significant negative correlation was detected between EDSS and IL-10 or Σ Th2 levels ($p=0.005$). In addition, IL-1 α ($r=0.4$, $p=0.05$) and IFN- γ ($r=0.35$, $p=0.05$) were also directly correlated with EDSS in female patients. **Conclusions:** Patients with RR-MS who are in the relapse clinical phase exhibit higher levels of pro-inflammatory cytokines and reduction in protective Th2-related cytokines.

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Keywords: Cytokines, Multiple Sclerosis, Th1, Th2, Th17

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INTRODUCTION

Multiple Sclerosis (MS) is an autoimmune disease in which the immune system mistakenly attacks the myelin sheaths around the axons of the brain and spinal cord leading to nerve conduction impairment (1). Active MS lesions are characterized by the infiltration of T cells and macrophages and the presence of their mediators (2,3). For a long time IFN- γ producing T helper type 1 (Th1) cells have been considered as the sole players in the pathogenesis of MS. However, the animal model of MS, experimental autoimmune encephalomyelitis (EAE), was very valuable to elucidate the key role of IL-17 producing Th17 cells in lesion development in CNS (4,5). Indeed, Th17 cells are found in the inflamed CNS of EAE mice and IL-17 is one of the cytokines that is intensely up-regulated in MS lesions (6). Conversely, cytokines produced by Th2 cells (especially IL-4) have been associated with remission from EAE (7,8) and ensuring a clinical response in MS patients to glatiramer acetate (9). However, this clear-cut immune-dysregulation of the Th1-Th17/Th2 balance in EAE and MS may be part of a hidden complex of interactions underlying EAE and MS (10). Regulatory T cells are involved in the regulation of Th1, Th2 and Th17 cells activity and have been shown to be vital for protection against EAE (11). In a number of studies it has been shown that the frequency of Treg cells does not differ between patients with MS and healthy controls (12), while Tregs are functionally compromised (13).

Given the fact that differentiated T cells (Th1, Th2, Th17 and Tregs) can perform their functions through cytokines production, the pattern of cytokines in the sera of MS patients have been investigated in a few studies (9, 14-18). The majority of these studies suffer from enrollment of a limited number of patients with various clinical forms of the disease during the remission and relapse phase. Therefore, the objective of the present study was to determine the levels of different pro-inflammatory and anti-inflammatory cytokines produced by innate immune cells (IL-1 α , IL-6, IL-8, IL-12, IL-15, IL-23, TNF- α), Th1 (TNF- β , IL-2, IFN- γ), Th2 (IL-4, IL-10, IL-13), and Th17 (IL-17) cells in relapsing-remitting MS (RR-MS) patients living in southwest of Iran compared to those in healthy subjects.

MATERIALS AND METHODS

Study Design and Subjects. Forty four RR-MS patients (12 males and 32 females) at relapse with an age range of 16-49 years were enrolled in this study. Patients diagnosed according to the McDonald 2010 criteria (19) by an expert neurologist. Forty one patients were new cases of RR-MS and the remaining 3 patients were not taken their medicines at least 3 months before sampling. MS patients who had taken any immunosuppressive medications within three months before sampling or had experienced any other autoimmune diseases were excluded from the study. For each eligible patient, the current EDSS score was determined by neurologist. Forty four gender- and age-matched healthy individuals without any underlying disease were selected as the control group. Control subjects showed neither clinical nor laboratory characteristics of autoimmune, infectious, or inflammatory diseases. The objective of the study was explained to all the participants and their written informed consents were obtained. This study was approved by the research ethics committee of Shiraz University of Medical Sciences (registration number 88-4151).

Cytokines Measurement. Sera were collected from all patients and controls and were stored at -80°C till examined. The levels of 15 cytokines were measured simultaneously using multiplexed human cytokine Strip wells 16-plex assay (Quansys Biosciences, San Diego, CA, USA) according to the manufacturer's instructions. The following cytokines were detected (detection limit is shown in parentheses): IL-1 α (3.64 pg/ml), IL-1 β (3.70 pg/ml), IL-6 (0.02 pg/ml), IL-8 (0.16 pg/ml), IL-12 (1.08 pg/ml), IL-15 (0.39 pg/ml), IL-23 (12.47 pg/ml), TNF- α (1.02 pg/ml), IL-2 (2.13 pg/ml), IFN- γ (1.80 pg/ml), IL-4 (1.37 pg/ml), IL-5 (1.55 pg/ml), IL-10 (0.25 pg/ml), IL-13 (0.01 pg/ml), and IL-17 (4.29 pg/ml). For multiplexed cytokine assay, thawed sera were gently vortexed and then centrifuged at $13200\times g$ for 10 min at 4°C immediately prior to testing. The principle of this assay is quantitative ELISA-based chemiluminescent allowing the concurrent measurement of different cytokines in 50 μl of samples. The chemiluminescence absorbance intensities were measured using the Q-View TM Imager and the concentrations of the relevant cytokines were determined for each analyte using standards provided by manufacturer and Q-View software and a log-log curve fit. The overall coefficient of variation (CV) for samples performed in duplicate was $<20\%$.

Statistical Analysis. The levels of cytokines were compared between cases and controls by non-parametric Mann-Whitney U test using Prism 6 software package (Graph Pad Software Inc., La Jolla, CA). Correlation statistics between serum cytokine levels and EDSS were calculated using Pearson Bivariate correlation test. A p-value of less than 0.05 was considered statistically significant. Moreover, for each variable the Mean \pm SEM and the range were also calculated.

RESULTS

Both the RR-MS patients and controls consisting of 12 males (%27.3) and 32 (%72.7) females were from the same ethnicity and geographic area (Southwest of Iran). In addition, patients and controls were age-matched (30.00 ± 1.20 years and 30.00 ± 1.40 years, respectively, $p=0.9$). The average age of disease onset was 28.41 ± 1.17 years in patients. After gender categorization, age at disease onset was 27.81 ± 0.12 in females and 30 ± 0.26 in male patients ($p=0.41$). Moreover, EDSS at the time of the study was 1.23 ± 0.18 . Sex classification revealed that female and male patients were not significantly different in EDSS (1.20 ± 0.02 and 1.29 ± 0.05 , respectively; $p=0.83$). However, bivariate correlation analysis showed significant positive correlation between EDSS and age of Patients ($r = 0.413$, $p=0.005$).

Comparison of Cytokines Levels between RR-MS Patients and Control Group. Serum levels of cytokines for RR-MS patients and control groups were determined strictly according to the manufacturer's guideline, utilizing recommended sample dilutions and standard curve concentrations. The serum concentrations of pro-inflammatory (IL-1 α , IL-1 β , IL-2, IL-6, IL-8, IL-12p70, IL-15, IL-17, IL-23, TNF α , and IFN- γ) and anti-inflammatory cytokines (IL-4, IL-10, and IL-13) are summarized in Table 1. The results were classified into innate-, Th1-, Th2-, and Th17-dependent cytokines as depicted in Figures 1-3 and Table 1.

Table 1. Cytokines levels in the sera of RR-MS patients and healthy controls.

Cytokine (pg/ml)	RR-MS (n=44) Mean ± SEM			Controls (n=44) Mean ± SEM			*P1	*P2	*P3
	M	F	T	M	F	T			
IL-1 α	1.5±1.3	2.0±0.7	1.9±0.6	6.0 ±3.2	3.0±1.1	3.7±1.1	0.8	0.3	0.4
IL-1 β	19±3.7	23.4±1.4	22.2±1.5	29.9±2.3	23.2±1.2	25.0±0.1	0.005	0.9	0.08
IL-2	4.8±4.8	4.1±1.6	4.3±1.7	11.8±5.0	1.5±1.1	4.3±1.7	0.22	0.15	0.9
IL-4	3.3±0.1	3.7±0.1	3.6±0.1	4.1±0.2	3.7±0.1	3.8±0.1	0.003	0.9	0.05
IL-6	8.7±5.4	4.8±2	25.9±2.2	1.3±0.7	0.5±0.3	0.7±0.3	0.3	0.005	0.003
IL-8	14.4±2.4	38.0±14.3	31.8±10.6	19.7±2.0	18.5±3.2	18.8±1.4	0.08	0.05	0.05
IL-10	2.6±2.6	13.6±6.2	10.7±4.7	91.7 ±33.9	39.6±14.0	53.0±13.8	0.05	0.3	0.02
IL-12	8.6±0.7	8.8±0.4	8.8±0.3	9.9±0.4	8.9±0.3	9.2±0.3	0.02	0.5	0.07
IL-13	7.3±0.5	8.4±0.5	8.1±0.4	9.0±0.5	7.7±0.4	8.1±0.3	0.05	0.5	0.7
IL-15	6.5±0.3	6.7± 0.2	6.6±0.2	7.1±0.4	6.4±0.2	6.6±0.2	0.13	0.7	0.7
IL-17	5.1±0.2	5.3± 0.1	5.2±0.1	5.7±0.2	5.4±0.2	5.5±0.1	0.16	0.4	0.14
IL-23	37.1 ±3.7	43.7±2.5	41.8±2.1	46.3±3.7	49.6±7.1	48.8±5.3	0.12	0.6	0.8
IFN- γ	13.8±6.6	11.7±3.4	12.3±3.0	15.6 ±2.5	4.6±1.0	7.4±1.2	0.17	0.2	0.9
TNF- α	37.4±5.0	30.2±3.7	32.2±3.0	26.6±2.3	18.4±1.1	20.7±1.2	0.13	0.005	0.002
TNF- β	5.6±0.4	6.2±0.4	6.0±0.3	9.2±0.43	6.2±0.3	7.0±0.3	<0.0001	0.7	0.02
Σ Th1*	24.1±8.5	47.6±25.8	41.2±18.9	41.4±6.8	12.3±1.8	20.3±3.0	0.04	0.05	0.9
Σ Th2*	37.2±23.9	34.2±11.7	35.4±10.3	128.9±39	451.0±14.1	72.3±15.5	0.007	0.8	0.05

* Σ Th1 is serum levels of IL-2 + IFN- γ + TNF- β ; Σ Th2 is serum levels of IL-4+IL-10+IL-13.

*p1, *p2 and *p3 were used for comparison of males (M), females (F), and total (T) cases and controls.

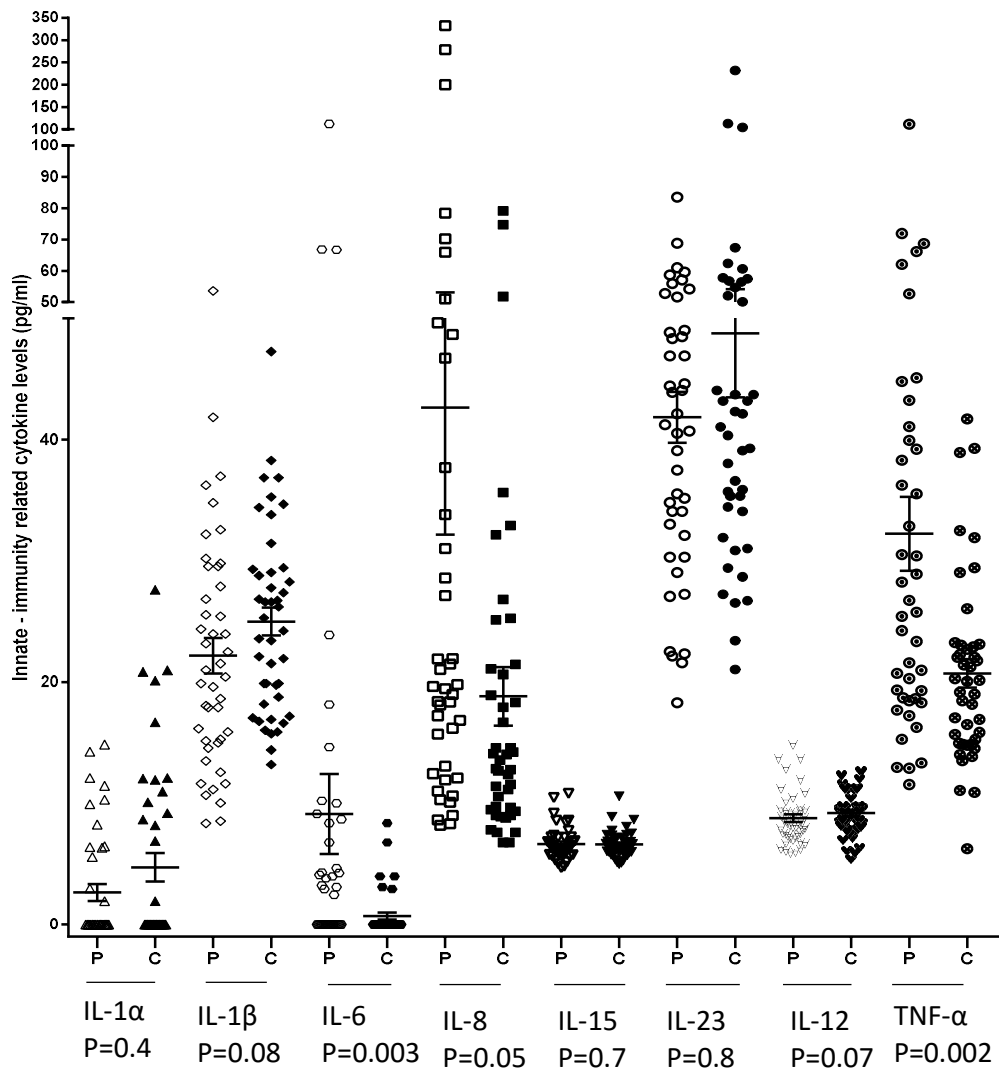


Figure 1. The levels of cytokines produced by innate immune cells in the sera of RR-MS patients (P) and controls (C).

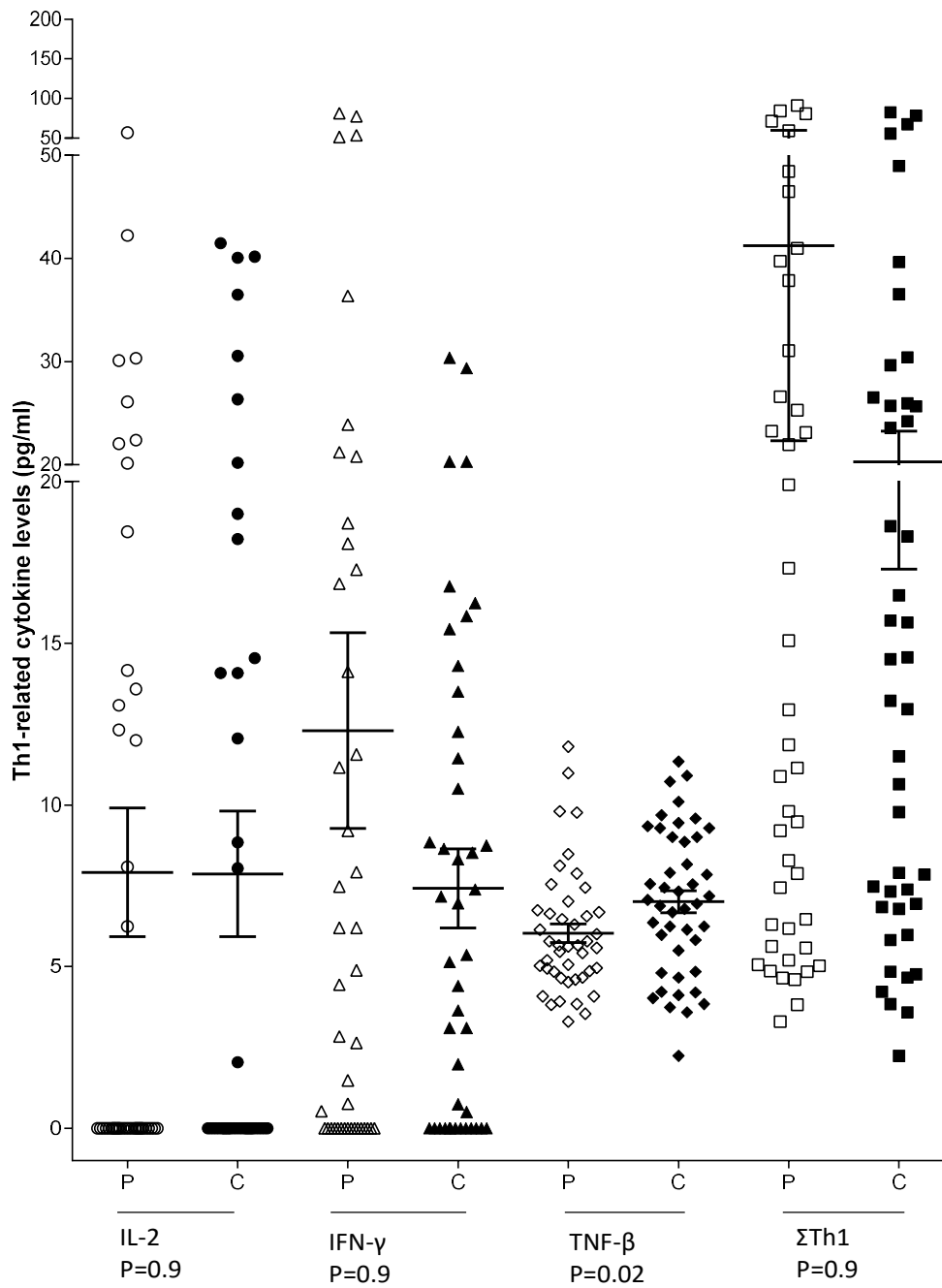


Figure 2. The levels of cytokines produced by Th1 immune cells in the sera of RR-MS patients (P) and controls (C).

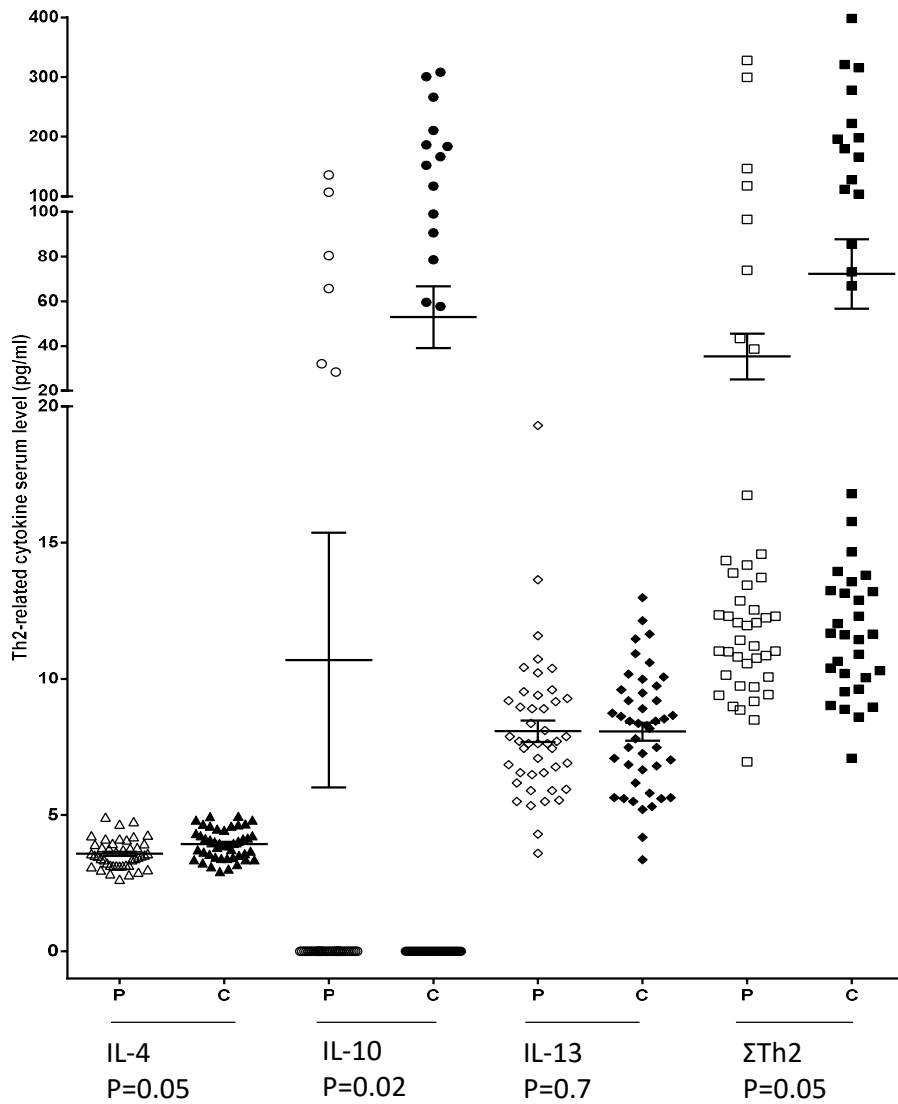


Figure 3. Serum levels of Th2-related cytokines in RR-MS patients (P) and control group (C).

As shown in Figure 1, comparison of serum levels of cytokines related to innate immunity in RR-MS patients and healthy controls showed significantly higher levels of IL-6 (5.91 ± 2.20 pg/ml and 0.69 ± 0.29 pg/ml, respectively; $p=0.003$) and TNF- α (32.23 ± 3.04 pg/ml and 20.71 ± 1.16 pg/ml, respectively; $p=0.002$) in the serum of patients compared to those of controls. The univariate analysis demonstrated that sera

levels of IL-1 α , IL-8, IL-12, IL-15, and IL-23 were not significantly different between RR-MS patients and controls ($p=0.4$, $p=0.14$, $p=0.07$, $p=0.7$ and $p=0.8$, respectively). Among Th1-dependent cytokines (Figure 2), TNF- β levels were significantly lower in RR-MS patients compared to controls (6.02 ± 0.29 and 7.01 ± 0.33 , respectively; $p=0.02$), while the mean serum levels of IFN- γ and IL-2 did not show any significant difference between cases and controls ($p=0.9$ and $p=0.94$, respectively). Also mean of Σ Th1 levels was not significantly different between RR-MS patients and controls ($p=0.9$). In addition, Th17-dependent cytokine (IL-17) did not show any significant difference between patients and controls ($p=0.14$). In the present study the Th2-associated cytokines as well as the Σ Th2-associated cytokines were compared between cases and controls (Figure 3, Table 1). Interestingly, the results revealed that while IL-4, IL-10 and Σ Th2-dependent cytokines (IL-4 + IL-10 + IL-13) were significantly higher in controls compared to those of patients suffering from RR MS ($p=0.05$, $p=0.02$ and $p=0.05$, respectively), serum levels of IL-13 were not significantly different ($p=0.7$). Of interest, after classification of cases and controls based on their gender, it was revealed that while the differences between IL-1 β , IL-12, and IL-13 were not significant between total cases and controls, the serum levels of these cytokines were significantly higher in male controls compared to those in male patients ($p=0.005$, $p=0.02$, and $p=0.05$, respectively; Table 1). In addition, subsequent to stratification on the basis of gender, only differences in the levels of IL-6 and TNF- α remained significant in females though the differences in TNF- β and IL-10 continued to be significant in males (Table 1).

Correlation of EDSS with other Variables in RR-MS Patients. Bivariate correlation analysis showed significant positive correlation between EDSS and age of RR-MS patients ($p=0.005$). Significant negative correlation between EDSS and the serum levels of IL-10 ($r=-0.414$, $p=0.005$) or Σ Th2-related cytokines ($r=-0.414$, $p=0.005$) was also detected. In addition, when patients were stratified according to their gender, the correlation of Σ Th2-related cytokines with EDSS only remained significant in female patients ($r=-0.55$, $p=0.001$) and IL-1 α ($r=0.4$, $p=0.05$) and IFN- γ ($r=0.35$, $p=0.05$) were also positively correlated with EDSS in female patients. No significant correlation was found between other cytokine levels and EDSS in MS patients.

DISCUSSION

As recently postulated, different MS subtypes can have different underlying disease mechanisms (14). Due to the fact that studies analyzing comprehensive cytokines profile in clinically defined patients with MS are limited, in the present study the serum cytokine profiles exhibited by RR-MS patients, the most common clinical form of MS, in relapsed phase was compared with age- and gender- matched controls in a population from Southwest of Iran. Results obtained in this study showed higher serum levels of pro-inflammatory cytokines (IL-6, IL-8 and TNF- α) and lower serum levels of anti-inflammatory cytokines (IL-4 and IL-10) in the sera of patients with RR-MS compared to those in controls. In addition, considering the pathogenic role of Th1 and Th17-dependent cytokines and possible protective role of Th2-dependent cytokines in MS pathogenesis, in the present study the summation of Th1- and Th2-related cytokines were also calculated to obtain a better understanding of cytokines imbalances which lead to multiple sclerosis. In this regard, serum levels of Th2 dependent cytokines (IL-4 and IL-10) as well as Σ Th2-related cytokines (IL-4+IL-10+IL-13) were significantly

lower in RR-MS patients compared to control subjects. These findings are in line with previous studies showing increased levels of Th2 cytokines are particularly evident during MS remission, whereas increased pro-inflammatory cytokines are found during relapse in MS patients (20-22). Therefore, the results of the present study support the hypothesis that an imbalance in the production of cytokines is involved in the pathogenesis of RR-MS. In fact, our results confirm and extend previous observations demonstrating that pro-inflammatory-related cytokines are elevated while the Th2-related cytokines are decreased in the blood of RR-MS patients especially during exacerbation (23,24). Among pro-inflammatory cytokines, unexpectedly the levels of TNF- β were significantly lower in RR-MS patients (Table 1). Of interest, gene expression studies in white blood cells from RR-MS patients in remission have shown an increased expression of TNF- β in remission compared to healthy controls (25). Therefore, one can conclude that TNF- β might be involved in activation of some unknown mechanisms leading to amelioration of MS. In this case, higher levels of TNF- β in the sera of healthy controls compared to RR-MS patients in relapse is not unexpected. IL-8 is another pro-inflammatory cytokine that showed significantly higher levels in the sera of RR-MS patients compared to controls (Table 1). Interestingly, it has been reported that IL-8 level is unequivocally elevated in the CSF during clinical relapse of MS (26). In addition, the association of single nucleotide polymorphisms in the gene of IL-8 with susceptibility to MS has also been reported (27). However, the mechanism by which IL-8 is involved in the pathogenesis of MS is not well defined. Moreover, after gender classification, while there were no significant differences in the levels of investigated cytokines between male and female patients, the levels of IL-1 β was only significantly higher in male controls compared to male patients with RR-MS (Table 1). It has been shown that IL-1 plays an important role in the protection of neurons and axons in the CNS of MS patients (28). Therefore, higher levels of IL-1 β in the sera of healthy male individuals compared to male RR-MS patients might be explained based on the role of this cytokine in induction of nerve growth factor and proliferation of nerve cells (28). Of interest, after gender stratification, male controls had significantly higher concentrations of anti-inflammatory cytokines (IL-4, IL-10 and IL-13) than did male patients with RR-MS (Table 1). The significant lower concentration of anti-inflammatory cytokines in male patients can explain the higher progression index in our set of male patients compared to female patients with RR-MS (medians: 29.5 and 19.8, respectively; $p=0.025$). However, the reason of gender-biased differences in the levels of mentioned cytokines is not known and needs further investigations. Finally, correlation analysis in this study revealed that the plasma levels of IL-10 and Σ Th2 were negatively associated with EDSS in patients suffering from RR-MS. This result shows that Th2-related cytokines may play an important role in decreased disease activity. In fact, the increased levels of IL-10 and Σ Th2-related cytokines in normal controls may lead to the decreased levels of pathogenic cytokines (IFN- γ and Σ Th1, Table 1), though the lesser levels of inflammatory cytokines in healthy controls did not reach the significant levels compared to the patients. Of interest, after classification of patients according to their gender, the Σ Th2 levels was still negatively correlated with EDSS in female patients ($r=-0.55$, $p=0.001$). Accordingly, higher levels of anti-inflammatory cytokines are probably involved in reducing the severity of RR-MS by inhibition of pathogenic Th1 and/or Th17 cells. It is somewhat surprising that no correlation was detected between the levels of inflammatory cytokines and EDSS (data not shown). However, after sex classification,

in female patients a positive correlation was found between EDSS and IL-1 α or IFN- γ . Due to the fact that these cytokines are among the pro-inflammatory cytokines, their positive correlation with EDSS in female patients is expectable. Considering the lower number of male patients (n=12) compared to female patients (n=32), gender biased correlations might be affected by the insufficient number of male patients. Therefore, before any conclusions it is necessary to increase the number of samples in order to increase the power of statistical test.

In summary, our results may indicate the higher levels of Th1-inducing cytokines and decreased levels of anti-inflammatory cytokines may play a significant role in the pathogenesis of RR-MS. In addition, reduced levels of IL-10 and Th2-related cytokines is correlated with increased EDSS. Accordingly, any therapeutic application for RR-MS should aim the induction of IL-10 and inhibition of Th1- and Th17- related responses.

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