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Altered Expression of Specific Transcription Factors of Th17 (RORγt, RORα) and Treg Lymphocytes (FOXP3) by Peripheral Blood Mononuclear Cells from Patients with Multiple Sclerosis

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Abstract The imbalance in Th17/Treg cell-related responses plays an important role in the pathogenesis of multiple sclerosis (MS). The development of Th17- and Treg cells is regulated by specific transcription factors—RORγt and RORα—and FOXP3, respectively. The aim was to determine the expression of RORγt, RORα, and FOXP3 in peripheral blood mononuclear cells (PBMCs) from MS patients following in vitro stimulation. The PBMCs from 22 MS patients and 20 healthy subjects were cultured in the presence of 10 μg/ml MOG, 10 μg/ml PHA, or without stimulation. The PBMCs were incubated at 37 °C for 24 h, and then the messenger RNA (mRNA) expression of RORγt, RORα, and FOXP3 was determined by real-time PCR. The expression of RORγt and RORα was increased in non-stimulated, MOG-stimulated, and PHA-stimulated PBMCs from MS patients in comparison with same cultures from the healthy group ($P < 0.01$, $P < 0.01$, and $P < 0.02$ for RORγt; $P < 0.001$, $P < 0.001$, and $P < 0.05$, for RORα, respectively). The FOXP3 expression in non-stimulated PBMCs from MS patients was significantly lower than that in equal culture from healthy subjects ($P < 0.05$). There were no significant differences between healthy subjects and MS patients regarding the expression of FOXP3 mRNA by MOG-stimulated and PHA-stimulated PBMCs. These results showed an imbalance in Th17/Treg cells at transcription factor levels with a deviation toward Th17 cell in MS. The correction of Th17/Treg balance at transcription levels should be considered to design novel therapeutic strategies for MS treatment.

Keywords Multiple sclerosis · Th17 · Treg · Transcription factors · RORγt · RORα · FOXP3

Introduction

Multiple sclerosis (MS) is an autoimmune-mediated demyelinating disease of the central nervous system (CNS) (Mahad et al. 2015). It seems that autoreactive $CD4^+$ T helper (Th) cells play an important role in the pathological process of MS (Kostic et al. 2015). Following the antigenic stimulation, various types of Th cells are differentiated from naïve T cells, such as Th1, Th2, Th17, or regulatory T (Treg) cells which were distinguished according to their cytokine patterns (Raphael et al. 2015). Both IFN-γ-producing Th1 cells and IL-17-releasing Th17 cells are involved in the development of MS and its animal model, experimental allergic encephalomyelitis (EAE) (Raphael et al. 2015; Volpe et al. 2015). The Treg cell-related cytokines (TGF-β and IL-10) were associated with reduction and improvement of MS and EAE symptoms (Buc 2013; Raphael et al. 2015). The role of Th2 cells, which secrete high levels of IL-4, IL-5, and IL-13, remains controversial (Raphael et al. 2015). We have previously observed the elevated concentrations of a Th17-related chemokine

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(CCL20), diminished levels of a Th2/Treg-related chemokine (CCL22), and unchanged levels of a Treg-related cytokine (IL-35) in patients with MS (Jafarzadeh et al. 2014a; Jafarzadeh et al. 2014b; Jafarzadeh et al. 2015).

Th17 cells play an important role in the host defense against some extracellular pathogens (such as *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, and *Candida albicans*) and participate in inflammatory reactions and autoimmune diseases (Rathore and Wang, 2016; Volpe et al. 2015). The differentiation of Th17 cells from naïve $CD4^+$ T cells is regulated by cytokines (including IL-6 and TGF-β), signal transducers (including STAT-3 and Smad), and transcription factors retinoic acid receptor-related orphan receptors (including RORγt and RORα) (Rathore and Wang, 2016; Volpe et al. 2015). The antigenic stimulation of naïve T cells in the presence of TGF-β and IL-6 results in the expression of the RORγt, through STAT-3 and Smad pathways. Both RORγt and RORα serve as important transcription factors regulating Th17 cell differentiation (Noack and Miossec 2014; Volpe et al. 2015). The deletion of both RORα and RORγt genes interrupt the differentiated of naïve $CD4^+$ T cells to Th17 lymphocytes. However, the deletion of RORγt gene exhibit stronger consequences on Th17 cell differentiation, suggesting that the RORγt is more important (Noack and Miossec 2014).

IL-23 is a cytokine inducing TH17 cell maintenance and full activation. After the initial differentiation of naïve $CD4^+$ T cells to Th17 cells, subsequent exposure to IL-23 is needed for the functional maturation and pathogenic action of Th17 cells (Floss et al. 2015; Rathore and Wang, 2016). Th17 cells are characterized by the secretion of several cytokines and chemokines including IL-17 (also called IL-17A), IL-17F, IL-21, IL-22, IL-23, IL-26, TNF-α, CCL20, and GM-CSF, although some of these cytokines are not Th17-specific (Basu et al. 2013; Rathore and Wang, 2016). IL-17 can influence different cells including endothelial cells, epithelial cells, fibroblasts, myeloid cells, and synoviocytes (Bedoya et al. 2013; Volpe et al. 2015). IL-17 induces the secretion of the various inflammatory mediators, including IL-8, CXCL1, CXCL6, IL-1β, IL-6, TNF-α, GM-CSF, MIP-2, and MCP-1 G-CSF (Bedoya et al. 2013; Volpe et al. 2015). The pathogenic role of Th17 cells as well as IL-17 was demonstrated in a number of autoimmune diseases such as MS, Crohn's disease, psoriasis, and rheumatoid arthritis (Volpe et al. 2015).

Treg cells play an important role in maintaining the peripheral tolerance. They can inhibit the function of other effector T cells and antigen-presenting cells by different mechanisms such as cell-cell contact and the secretion of suppressive cytokines TGF-β, IL-10, and IL-35 (Rodriguez-Perea et al. 2016). The dysfunction of Treg cells can result in a number of autoimmune diseases such as SLE and RA (Nie et al. 2015).

Treg cells comprise approximately 5–15 % of the $CD4^+$ T cells and were divided into two subgroups, including natural Treg (nTreg) and inducible Treg (iTreg) cells. The nTreg cells are generated from precursor cells in the thymus and iTreg cells are differentiated from naïve T cells at peripheral tissues in the presence of IL-2 and TGF-β (Noack and Miossec 2014; Rodriguez-Perea et al. 2016). In the periphery, antigenic stimulation of naïve $CD4^+$ T cells in the presence of TGF-β and IL-2 results in the differentiation of naïve cells into iTreg cells (Noack and Miossec 2014).

Both nTreg and iTreg cells express master transcription factor FOXP3 which is encoded by the *foxp3* gene on the X chromosome (Nie et al. 2015; Noack and Miossec 2014). The Treg cell activity was regulated by the transcription factor FOXP3, and the mutations of the *foxp3* gene disturb the function of Treg cells, therefore resulting in the development of various autoimmune diseases (Nie et al. 2015; Noack and Miossec 2014). The scurfy mice strain exhibit a mutation in the *foxp3* gene, which results in the abnormal expression of FOXP3. Many mutations in the *foxp3* gene have also been reported in patients with IPEX (immune-dysregulation, polyendocrinopathy, enteropathy, and X-linked inheritance syndrome) (Nie et al. 2015).

The balance of Th17/Treg cells regulates the immune activities and there some evidence suggesting that Th17/Treg imbalance contributes to the development of various autoimmune diseases (Noack and Miossec 2014). The results of a number of investigations were demonstrated that Th17 cells were increased and the numbers and/or function of Treg cells were decreased in the peripheral blood of MS patients, representing an important role of Th17/Treg cell imbalances in the pathogenesis of MS (Noack and Miossec 2014). However, the molecular mechanisms that influence the Th17/Treg imbalances in MS remain unknown.

The measurement of the transcription factors RORγt, RORα, and FOXP3 will more objectively reflect Th17/Treg cell differentiation than the simple measurement of a single Th17- or Treg cell-related parameter, alone (Lin et al. 2015). Thus, it is more important to investigate the balance of Th17 and Treg cells at their transcription factor level. Although there are some reports regarding the transcription factors in the MS patients or in EAE models (Edstrom et al. 2011; Martinez et al. 2014), there is no investigation concerning the measurement of these parameters in PBMCs from MS patients following the specific and non-specific in vitro stimulation. Thus, this study was conducted to determine the messenger RNA (mRNA) expression of specific transcription factors of Th17- (RORγt, RORα) and Treg cells (FOXP3) by PBMCs from newly diagnosed patients with MS following specific stimulation with MOG, non-specific stimulation with PHA, or without stimulation to provide new insights regarding the MS immunopathogenesis.

Material and Methods

Subjects

Blood samples were collected from 22 MS patients (age 35.56 ± 11.16 years; 8 men and 14 women) referring to the Shephah Hospital of Kerman (a city located in southeast of Iran). The patients were in the RRMS courses of disease. The presence of MS was confirmed by expert neurologists according to the clinical and paraclinical findings (MRI study, oligoclonal bands in CSF, and evoked potentials) based on McDonald's criteria (McDonald et al. 2001). All patients were newly diagnosed and none of them received treatment before blood sample collection.

In total, 20 healthy subjects (age 36.45 ± 11.73 years; 8 men and 12 women) were also enrolled in the study as a control group. The healthy individuals were recruited among blood donors of the Blood Transfusion Organization of Kerman and interviewed concerning the CNS disorders and none of them had CNS or other relevant disease. All control subjects were basically in good health, with no acute or chronic illnesses. Those with diseases (including a history of recurrent infections, asthma, allergy and atopic diseases, any suspected immunological diseases, and malignancy), smoking, medication, surgery, and major trauma within 6 months prior to blood collection were all excluded from the investigation.

This study evaluated and approved by the Ethical Committee of Kerman University of Medical Sciences. Moreover, participants were enrolled into the study if they agreed and informed written consent was also obtained from all of them. A peripheral blood sample (5 ml) was obtained from all participants, and the PMBCs were separated for more analysis.

In Vitro Stimulation of PBMCs

The PBMCs were isolated from heparinized peripheral blood by gradient centrifugation over Lymphosep (Biosera, UK). The separated PBMCs were washed three times and then were resuspended in a RPMI-1640 medium (Gibco Life Technologies Ltd, Paisley, UK) supplemented with 10 % heat inactivated fetal bovine serum (Gibco Life Technologies Ltd, Paisley, UK) and antibiotics, including penicillin (100 U/ml) and streptomycin (100 μg/ml). The cells were then cultured at 1×10^6 cells/ml in 24-well sterile flat-bottomed microtiter plates (Nunc, Thermo Fisher Scientific Inc, Denmark) in the presence of 10 μg/ml MOG [(35–55) human; Anaspec, USA], 10 μg/ml phytohemagglutinin mitogen (PHA) (Gibco Life Technologies Ltd, Paisley, UK)] as positive control or without any stimulation as negative control. The PBMCs were cultured at 37 °C in a 5 % CO_2 incubator for 24 h. After this period, the PBMCs were harvested for total RNA extraction.

RNA Extraction, Reverse Transcription, and Quantitative Real-Time PCR

Total RNA was extracted from cultured PBMCs by using the Trizol reagent (Bionner, Korea) according to the manufacturer's guidelines. To prevent the amplification of contaminating genomic DNA, extracted RNA was treated with DNase I (Thermo Scientific, EU). The quality and purity of the extracted RNA was determined by electrophoresis on an ethidium bromide pre-treated agarose gel together with the measurement of absorption by spectrophotometer and calculation of 260/280 ratio. The RNA was converted to complementary DNA (cDNA) using a cDNA synthesis kit (Bionner, Korea) with both oligo (dT) and random hexamer primers. The expression of the RORγt, RORα, and FOXP3 in the cultured PBMCs was determined by real-time PCR. The β-actin gene was used as a housekeeping control. The primers used are demonstrated in Table 1.

The reverse transcription was performed according to the following protocol: 70 °C for 10 min (without reverse transcription enzyme), −20 °C for 1 min (cooling step), addition of reverse transcription enzyme, 42 °C for 60 min, and the protocol was finished by a final step at 95 °C for 10 min to halt the activation of the reverse transcription enzyme.

The real-time PCR method was performed by using a SYBR green master mix (Bionner, Korea), combined with 200 ng of template cDNA with the appropriate primers (Table 1) in a real-time PCR System (Applied Biosystems, USA) using the following program: 1 cycle of 95 °C for 15 min, 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and finally 72 °C for 30 s. Primers were synthesized by the Bionner company (Korea). The relative mRNA expression of transcription factors was normalized by β actin as a housekeeping gene and calculated by the $2^{-\Delta\Delta Ct}$ formula. The melting

Table 1 The used primers for the gene expression of RORγt, RORα, and FOXP3 by PBMC from healthy subjects and MS patients

Gene	Primer
RORγt	Forward: 5-TGGAAGTGGTGCTGGTTAGGATG-3
	Reverse: 5-GGAGTGGGAGAAGTCAAAGATGGA-3
RORα	Forward: 5-CTACATTGACGGGCACACC-3
	Reverse: 5-ACACAGTTGGGGAAGTCTCG-3
FOXP3	Forward: 5-AGAAGCAGCGGACACTCAAT-3
	Reverse: 5-CACTTGTGCAGACTCAGGTTGT-3
β-Actin	Forward: 5-GCATGGGTCAGAAGGATTC-3
	Reverse: 5-GTCCCAGTTGGTGACGAT-3

curves and quantitative analyses of the data were performed using Applied Biosystems software version 1.1.308.111 (USA). PCR products were also analyzed by electrophoresis and visualized on a 1 % agarose gel containing 0.5 mg/ml ethidium bromide.

Statistical Analysis

Data are presented as mean ± SEM. Statistical analysis was performed by using ANOVA, *t* test, Kruskal-Wallis, and Mann-Whitney *U* tests, as appropriate. The *P* values of less than 0.05 were considered statistically significant.

Results

The Expression of Th17 Cell-Specific Transcription Factor RORγt by PBMCs

The fold change expression of transcription factors, by non-stimulated, MOG-stimulated, and PHA-stimulated PBMCs from healthy subjects and MS patients are demonstrated in Table 2.

In the healthy control group, the mRNA expression of RORγt in PHA-stimulated PBMCs was significantly higher than those in MOG-stimulated and non-stimulated PBMCs ($P < 0.03$ and $P < 0.01$, respectively). No significant difference was observed between non-stimulated and MOG-stimulated PBMCs, regarding the mRNA expression of RORγt, although this parameter was higher in MOG-stimulated PBMCs (Table 1).

In MS patients, the expression of RORγt mRNA was significantly up-regulated in PHA- and MOG-stimulated PBMCs in comparison with non-stimulated PBMCs ($P < 0.01$ and $P < 0.04$, respectively). There were no significant differences between PHA-stimulated and MOG-stimulated PBMCs regarding the gene expression of RORγt, although this parameter was higher in a PHA-stimulated culture (Table 1).

The mRNA expression of RORγt in non-stimulated, MOG-stimulated, and PHA-stimulated PBMCs from MS patients was significantly higher than that in counterpart cultures from the healthy control group ($P < 0.01$, $P < 0.01$, and $P < 0.02$, respectively) (Fig. 1).

The Expression of Th17 Cell-Specific Transcription Factor RORα by PBMCs

In the healthy group, the expression of RORα mRNA in PHA-stimulated PBMCs was significantly increased as compared with MOG-stimulated and non-stimulated PBMCs ($P < 0.001$ and $P < 0.001$, respectively). There was no significant difference between non-stimulated and MOG-stimulated cultures, with respect to the expression of RORα, although this parameter was higher in MOG-stimulated PBMCs.

In patients with MS, the expression of RORα mRNA was significantly up-regulated in PHA- and MOG-stimulated PBMCs in comparison with non-stimulated PBMCs ($P < 0.04$ and $P < 0.04$, respectively). In MS patients, the expression of RORα mRNA was also significantly up-regulated in PHA-stimulated PBMCs in comparison with a MOG-stimulated culture ($P < 0.05$) (Table 1).

The expression of RORα in non-stimulated, MOG-stimulated, and PHA-stimulated PBMCs from MS patients were significantly higher than those in the same cultures from the healthy control group ($P < 0.001$, $P < 0.001$, and $P < 0.05$, respectively) (Fig. 2).

The Expression of Treg Cell-Specific Transcription Factor FOXP3 by PBMCs

In the healthy group, the PHA-induced expression of FOXP3 in PBMCs was significantly higher than non-stimulated and MOG-induced cultures ($P < 0.03$ and $P < 0.04$, respectively). There was no significant difference between the non-stimulated and MOG-stimulated PBMCs, concerning the expression of FOXP3 (Table 2).

In MS patients, the expression of FOXP3 in MOG-stimulated and PHA-stimulated cultures were significantly higher than those in non-stimulated PBMCs ($P < 0.01$ and $P < 0.01$, respectively). The PHA-induced expression of FOXP3 was also significantly increased as compared with MOG-stimulated PBMCs ($P < 0.05$) (Table 2).

The expression of FOXP3 mRNA in non-stimulated PBMCs from MS patients was significantly lower than that

Table 2 The expression of RORγt, RORα, and FOXP3 by PBMC from healthy subjects and MS patients

Groups	Stimulator of PBMC	RORγt expression	RORα expression	FOXP3 expression
Healthy group	Without stimulation	1.00 ± 0.36	1.00 ± 0.44	1.00 ± 0.45
	MOG	2.26 ± 0.96	1.32 ± 0.53	0.87 ± 0.33
	PHA	5.10 ± 1.04	15.29 ± 2.91	7.54 ± 3
MS patients	Without stimulation	3.66 ± 1.18	11.55 ± 4.17	0.24 ± 0.16
	MOG	11.23 ± 3.32	29.42 ± 7.59	1.36 ± 0.30
	PHA	15.81 ± 4.43	68.06 ± 17.57	4.70 ± 1.67

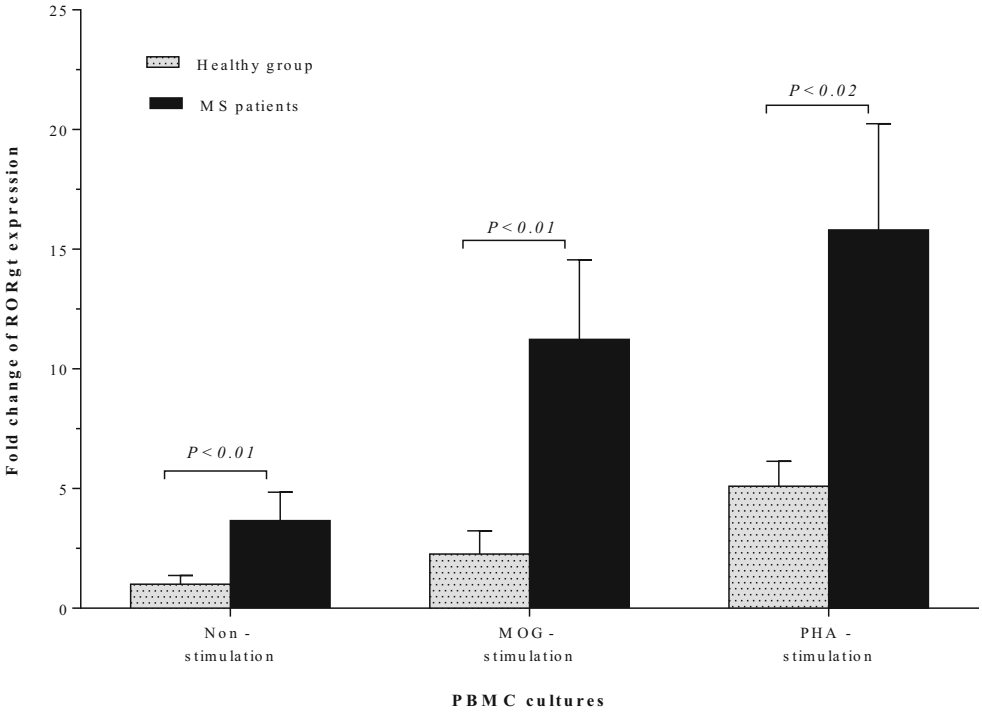

Fig. 1 Comparison of the mRNA expression of Th17 cell-specific transcription factor RORγt by PBMC from healthy subjects and MS patients

in equal culture from the healthy control group ($P < 0.05$) (Fig. 3). The differences in the expression levels of FOXP3 mRNA by MOG-stimulated and PHA-stimulated PBMCs between healthy subjects and MS patients were not statistically significant (Fig. 3).

Discussion

The results of this study showed that in both healthy subjects and MS patients, the expression of RORγt, RORα, and FOXP3 in PHA-stimulated PBMCs was significantly

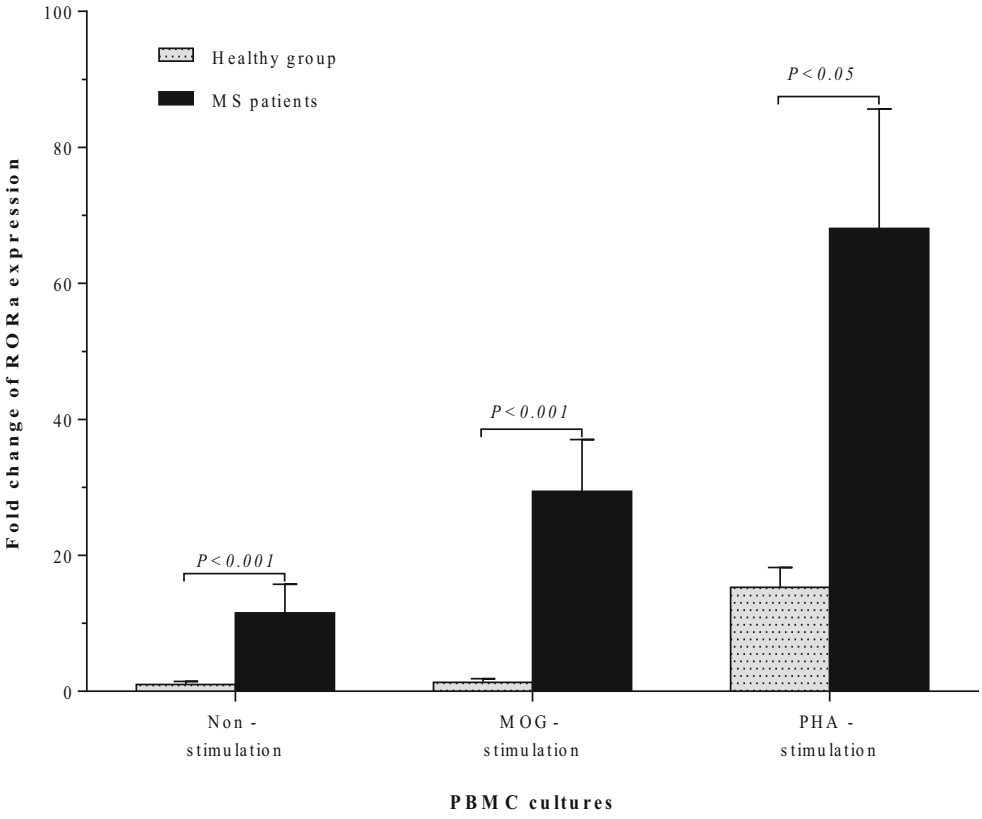

Fig. 2 Comparison of the mRNA expression of Th17 cell-specific transcription factor RORα by PBMC from healthy subjects and MS patients

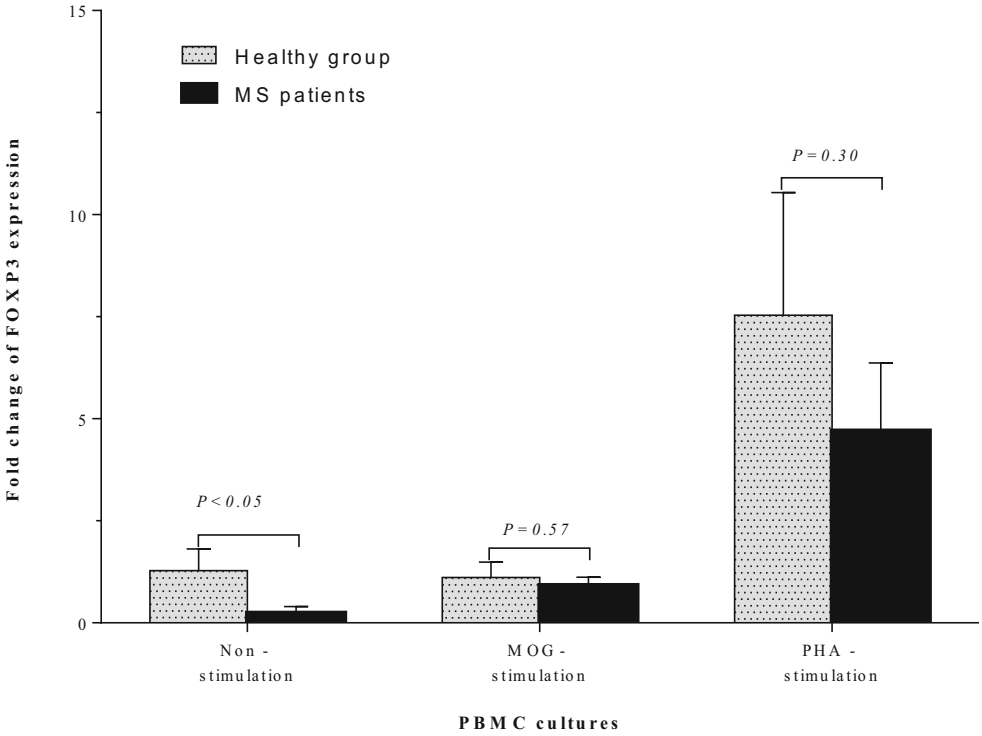

Fig. 3 Comparison of the mRNA expression of Treg-specific transcription factor FOXP3 by PBMC from healthy subjects and MS patients

enhanced as compared with non-stimulated cultures. The PHA was used as a positive control inducer, and its effects can be ascribed to the polyclonal activation of T cells, which induces the differentiation of naïve $CD4^+$ T cells into various effector T cells including Th17 and Treg cells. In healthy subjects, no significant difference was observed between non-stimulated and MOG-stimulated PBMCs, regarding the expression of RORγt and RORα, whereas in MS patients these parameters were increased in MOG-stimulated cultures. This difference may be due to the prior in vivo sensitization of T cells to myelin antigens (such as MOG) in MS patients.

The expression of RORγt and RORα in non-stimulated, MOG-stimulated, and PHA-stimulated PBMCs from MS patients were significantly higher than those in the same PBMC cultures from the healthy control group. RORγt and RORα are two transcription factors directly mediating Th17 differentiation. Indeed, RORα in a synergic manner with RORγt enhances IL-17A production (Wang et al. 2013). Our findings represent that the frequency of MOG-specific Th17 cells in MS patients may be higher than that in controls. In accordance with our findings, high frequencies of autoreactive Th17 cells have been indicated in the CNS of MS patients and EAE mice (Becher and Segal 2011). The high IL-17 levels in the cerebrospinal fluid (CSF) of patients with MS also represent the involvement of this cytokine in the pathogenesis of disease (Kostic et al. 2014). Furthermore, RORγt deficiency leads to more resistance to EAE induction (Raphael et al. 2015). It has been also indicated that the IL-17-deficient mice display a delayed onset, lower EAE scores, and lower histological alterations with early recovery from disease (Raphael et al. 2015). Moreover, the higher expression of RORγt and RORα by PHA-stimulated PBMCs from MS patients represent an elevated capacity to generate Th17 cell-related immune responses in these subjects.

The possible mechanisms by those IL-17 is involved in immunopathogenesis of MS may be through the recruitment of neutrophils into the inflammatory sites, induction of the reactive oxygen species (ROS) production in brain endothelial cells, activation of microglia cells to produce pro-inflammatory cytokines and mediators, and the stimulation of astrocytes to produce CXC chemokines (Volpe et al. 2015). Elevated expression of matrix metalloproteinases (such as MMP-2, MMP3, MMP-7, and MMP-9) have been also reported in the CNS of MS patients (Mirshafiey et al. 2014). Some TH17 cell-derived cytokines (such as TNF-α) trigger the expression of matrix metalloproteinases, which may have a crucial role in the rupture of blood brain barrier (BBB) (Mirshafiey et al. 2014; Volpe et al. 2015).

The results of the present study showed that the expression of FOXP3 in non-stimulated PBMCs from MS patients was significantly lower than that in the same culture from the healthy control group. FOXP3 plays a fundamental role in the differentiation of the Treg cells while suppressing the development of Th17 cells (Noack and Miossec 2014). These findings were indicated that the downregulation of Treg cell-related immune responses may contribute to the MS

pathogenesis. No significant differences were observed between healthy subjects and MS patients regarding the expression of FOXP3 by MOG-stimulated and PHA-stimulated PBMCs. These findings may represent that there is no intrinsic defect in Treg cells in MS patients. It seems that the in vivo conditions in MS patients may have suppressive effects on the Treg cell activity. Therefore, in a culture environment, the inhibitory effects of in vivo conditions on Treg cell function may be eliminated and they will normally respond to in vitro antigenic stimulation. Interestingly, it has been demonstrated that the plasma exchange can increase the FOXP3/RORγt expression in patients with MS which is in accordance with our results (Jamshidian et al. 2015).

The results of this study also showed that in MS patients, the expression of FOXP3 in MOG-stimulated culture was significantly higher than that in non-stimulated PBMCs. Accordingly, the normal activity was exhibited by Treg cells after in vitro stimulation that represent that the extrinsic parameters may influence the Treg cell activity in the MS patients. There are some investigations concerning the number and/or function of Treg cells in MS patients with controversial results. Both decreased and unchanged in the number of Treg cells are reported in the peripheral blood of MS patients (Kostic et al. 2015). A number of in vitro studies have reported the impaired immunosuppressive function of Treg cells. It has been also reported that Treg cells isolated from MS patients had low inhibitory effects on myelin antigen-induced specific T cell proliferation in comparison with healthy subjects. Moreover, Treg obtained in the relapse phase of the MS exhibit impaired suppressor function, whereas Tregs collected during the remission phase display normal immunosuppressive activity (Shichita et al. 2009). These discrepancies may be largely attributed to the heterogeneity of Treg cell population, using of different markers of Treg cells in the studies and different inclusion criteria for MS patients.

The existence of the balance between Th17 and Treg cell-related immune responses are very important for maintaining the normal immunological functions. The occurrence of the imbalance between Th17/Treg cell-related immune responses may cause a number of disorders such as cancer, autoimmunity, allergy, and infectious diseases (Zhang et al. 2014). The results of the present study indicate that there is an imbalance in Th17/Treg cells at transcription factor levels with a tendency toward Th17 cell-related parameters in MS patients which may contribute to the pathogenesis of disease.

It should be noted that Th17 and Treg cells have opposite activities in the development of autoimmune and inflammatory diseases. The Th17 cells tend to potentiate autoimmunity, whereas Treg cells control it and therefore play a very important preventive role against autoimmunity by controlling the activation of autoreactive $CD4^+$ T effector cells (Noack and Miossec 2014). The differentiation process of Th17 and Treg cells is nearly related. TGF-β induces the expression of both FOXP3 and RORγt in naïve $CD4^+$ T cells, but the presence or the absence of IL-6 will be determined by the differentiation of naïve T cells toward Treg or Th17 cells. The antigenic stimulation of naïve $CD4^+$ T cells in the presence of TGF-β alone (in the absence of IL-6) result in the expression of FOXP3 whereas the expression of RORγt is suppressed; therefore, Treg cell-related response is dominant (Ivanova and Orekhov, 2015; Murphy and Stockinger, 2010). TGF-β is not capable to initiate the Th17 differentiation unless IL-6 is present. When IL-6 is present, the TGF-β-induced FOXP3 expression is reduced and RORγt expression is up-regulated (Ivanova and Orekhov 2015; Noack and Miossec 2014). Higher levels of IL-6 have been reported in patients with MS (Chen et al. 2012), which may be responsible for the Th17/Treg imbalance in these subjects. The Th17 and Treg cells also antagonize each other functionally. For instance, FOXP3 suppresses RORγt activation by a physical interaction while IL-6/STAT3 axis suppresses FOXP3 expression (Ichiyama et al. 2008).

As mentioned, the Th17/Treg imbalance was associated with the development and progression of MS. The correcting of Th17/Treg imbalance in MS may consider for designing of novel and effective immunotherapies which can be done at different levels such as cytokines, receptors, or signaling pathways. The STAT3 and STAT5 are two signaling factors which control the differentiation of Th17 and Treg cells, respectively. It has been reported that the inhibition of STAT3 by siRNA significantly decreases the proportion of Th17 cells and increases the proportion of Treg cells, among the $CD4^+$ T cell population from patients with rheumatoid arthritis (Noack and Miossec 2014). Similar immunotherapeutics by targeting transcription factors such as RORγt, RORα, and FOXP3 can present novel therapeutic strategies for the control of inflammation in MS patients.

In conclusion, the results of the present study indicated that the expression of RORγt and RORα were increased in non-stimulated, MOG-stimulated, and PHA-stimulated PBMCs from MS patients. However, the expression of FOXP3 mRNA was decreased in the non-stimulated PBMCs from MS patients. There were no significant differences between healthy subjects and MS patients regarding the expression of FOXP3 mRNA by MOG-stimulated and PHA-stimulated PBMCs. These results indicate an imbalance of Th17/Treg cells at transcription factor levels and a Th17 cell biased pattern in MS patients. The correction of Th17/Treg cells imbalance at transcription factor levels may consider in designing of the novel therapeutic strategies for treatment of MS in the future investigations.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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