Downregulation of Immunosuppressive Molecules, PD-1 and PD-L1 but not PD-L2, in the Patients with Multiple Sclerosis

Mohammad Reza Javan[1,2], Saeed Aslani[3], Mohammad Reza Zamani[3,4], Javad Rostamnejad[5], Milad Asadi[6], Mahdi Farhoodi[7], and Mohammad Hossein Nicknam[3, 8]

[1] Department of Immunology, Faculty of Medicine, Zabol University of Medical Sciences, Zabol, Iran
[2] Department of Immunology, School of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran
[3] Department of Immunology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
[4] Network of Immunity in Infection, Autoimmunity and Malignancy (NIIMA), Universal Scientific Education and Research Network (USERN), Tehran, Iran
[5] Department of Genetics, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[6] Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[7] Department of Neurology, Neurosciences Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[8] Molecular Immunology Research Center, Tehran University of Medical Sciences, Tehran, Iran

ABSTRACT

Programmed cell death-1 (PD-1) and its ligands, PD-L1 and PD-L2, have been regarded as important immune system regulatory molecules. The aberrant expression of the molecules has been related to several autoimmune disorders. This study is aimed to assess the mRNA expression level of PD-1, PD-L1, and PD-L2 molecules in the peripheral blood mononuclear mells (PBMCs) from multiple sclerosis (MS) patients.

PBMCs were isolated from the whole blood of 50 MS and 50 healthy individuals. Total RNA content of the leukocytes was extracted. Then, cDNA was synthesized from the extracted RNA. Afterwards, quantitative analysis of PD-1, PD-L1 and PD-L2 was carried out through Real Time PCR using the TaqMan gene expression assays.

Relative expression of PD-1 and PD-L1 in PBMCs from MS patients was significantly lower compared with the healthy control group (p=0.003 and 0.012, respectively). However, no significant difference was observed in the expression level of PD-L2 between patients and healthy individuals. Relative expression of PD-1 correlated with expanded disability status scale score (EDSS) of the patients (r=-0.763, p=0.008).

Downregulation of the immunosuppressive molecules, PD-1 and PD-L1, may imply that over-activation of immune cells in multiple sclerosis occurs through signaling dysfunction of these molecules and PD-L2 plays no important role in this context.

Keywords: Gene expression; Multiple sclerosis; Programmed cell death 1 receptor; Programmed cell death 1 ligand 1 protein; Programmed cell death 1 ligand 2 protein

Corresponding Author: Mohammad Hossein Nicknam, MD, PhD; Department of Immunology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran. Tel: (+98 21) 8895 3009, Fax: (+98 21) 6641 9536, E-mail: nicknam_m@yahoo.com

INTRODUCTION

Programmed cell death (PD-1) also known as CD279 is a novel negative regulatory molecule that belongs to the CD28/CTLA-4 family.[1] This molecule is expressed on activated $CD4^+$ and $CD8^+$ T cells, and binds to two known ligands, PD-L1 (B7- H1; CD274) and PD-L2 (B7-DC; CD273), found on antigen presenting cells (APCs) as well as on various parenchymal cell types.[2,3] The co-stimulatory pathway molecules including the PD-1 receptor (CD279) and its ligands, PD-L1 as well as PD-L2, provide inhibitory signals resulting in regulation of the balance among T-cell activation, tolerance, and immune-mediated tissue damage.[4] The role of PD-1/PD-L pathway in the prevention of autoimmune diseases has become more and more clear recently.[5] Through experimental models of autoimmunity and pathogenetic polymorphisms, it has been demonstrated that altered function of PD-1 and its ligands are associated with several human autoimmune conditions.[5]

Multiple sclerosis (MS) is an autoimmune disease and the most common chronic demyelinating disorder of the central nervous system (CNS) and is categorized as an immune-mediated inflammatory disease leading to substantial disability in the patients.[6] Even though the prevalence is approximately 0.1% in the Europe and United States, MS is highly distributed among young adults with the age range of 20 to 45 and imposes considerable economic burden.[7] The pathogenesis is a complex interaction between genetic, autoimmune and environmental factors.[8] However, it seems that either anti- or pro-inflammatory mediators secreted by different subtypes of helper T as well as other immune cells determines a manifestation of harmful or protecting immune response in MS.[9]

MS usually falls into four major groups based upon the duration of disease: relapsing remitting (RRMS), secondary progressive (SPMS), primary progressive (PPMS), and progressive relapsing (PRMS).[10] The disease frequently follows a relapsing and remitting pattern in its early stages, where patients experience symptomatic attacks or exacerbations which occurs for a period of time and then resolves. This type of MS occurs in 80–85% of patients and is known as RRMS.[11,12]

The interactions between genetic, epigenetic and environmental risk factors have been regarded as determinant elements for the clinical phenotype of the various autoimmune disorders.[13] The microRNA machinery component, as an epigenetic modification mechanism, has been reported to be dysregulated in MS.[14] Moreover, a PD-1 polymorphism has been associated with the progressive form of MS.[15] As a negative feedback approach to ameliorate the disease manifestations, PD-L1 has been observed to be up-regulated in MS lesions leading to down-regulation of T cell responses.[16] Considering the different mechanisms affecting autoimmune diseases and plausible importance of immunosuppressive molecules in MS, in this study for the first time, we evaluated the mRNA expression level of PD-1, PD-L1 and PD-L2 as immunosuppressive molecules in peripheral blood mononuclear cells (PBMCs) obtained from MS patients using real time PCR method.

PATIENTS AND METHODS

Participants

In this study, gene expression analysis was performed on a total of 100 individuals including 50 unrelated RRMS patients and 50 healthy individuals. RRMS Patients were chosen from individuals referred to the Neurosciences Research Center, Imam Reza Hospital, Tabriz University of Medical Sciences, Tabriz, Iran over the course of 2013-2014. None of the healthy participants had autoimmune disease neither in themselves nor their immediate family members and they were age and sex matched with patients. Diagnosis of MS in the patients was performed according to the principles described by modified McDonald criteria.[17,18] MS patients were in the remitting state. The patients had received no immunomodulatory therapy for at least 3 months before they included in the study. This study has been approved by human research ethics committee of Tabriz University of Medical Sciences (No. 1/2/9591). Written informed consent was taken from all participants. Blood samples from MS patients were obtained during clinical diagnosis; as such samples were obtained from healthy controls. About 10 ml of blood from each subject was collected in EDTA contained tubes.

PBMC Isolation and RNA Extraction

In order to isolate PBMCs from peripheral blood of the subjects, Ficoll-Hypaque density gradient centrifugation approach was exploited. Total cellular RNA was extracted using High Pure RNA Isolation Kit

(Roche, Germany) according to the manufacturer's manuals. The yield and purity of RNA were analyzed using a Nano Drop spectrophotometer at 260/280 nm (Nano Drop ND-2000C Spectrophotometer, Thermo Fisher Scientific, USA).

Reverse Transcription and Complementary DNA Synthesis

First-strand complementary DNA (cDNA) was synthesized from the cellular RNA e using the RevertAid First Strand cDNA Synthesis Kit (Fermentas, USA; Cat No. K1622), according to the manufacturer's instruction. Briefly, first 4 μl of the isolated RNA (30 μg) through the previous procedure were mixed with 1 μl of random hexamer primer and 7 μl RNase-free H_2O and then incubated at 65°C for 5 minutes. Afterwards, micro-tubes were chilled on ice, and a 4 μ of reaction buffer l, 1 μl RNase inhibitor, 2 μl dNTP mix and 1 μl reverse transcriptase was added to each sample. Samples were immediately incubated at 25°C for 5 minutes followed by 42°C for 60 minutes; the reaction was finally terminated by heating at 70°C for 5 minutes. Reverse transcription was performed with the final volume of 20 μL pre tube.

Real-Time Quantitative Polymerase Chain Reaction

Quantitative analysis was carried out by real time PCR (RT-PCR) using TaqMan Gene Expression Assays containing FAM dye-labeled probes (TaqMan Pre-designed Gene Expression Products, Applied Biosystems, Foster City, CA, USA) and StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Each reaction mixture contained a total volume of 25μl (master mix 12.5μl, cDNA 4.5μl, assay mix 3μl, and H_2O 5μl). The quantitative RT-PCR conditions were: 50°C for 2 minutes, 95°C for 10 minutes, then 40 cycles of 95°C for 30 seconds, and 60°C for 30 seconds and 72°C for 30 seconds. A widely used approach to represent relative gene expression, namely the comparative C_T method ($2^{\Delta\Delta Ct}$ method), was exploited to evaluate expression as previously described by Livak and Schmittgen.[19] Relative amounts of target mRNAs in the test samples were calculated and normalized to the corresponding β-Actin mRNA transcript level as a housekeeping gene.

Statistical Analysis

Data analysis was performed through SPSS software version 21 (SPSS, Chicago, IL, USA). Scale variables were calculated for normality using the Kolmogorov-Smirnov test. Through the independent sample *t*-test, group comparisons of continues variables were carried out. If the variable was not normally distributed, Mann-Whitney nonparametric test was conducted. The GraphPad Prism version 5.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com) was applied to illustrate data through graphs. All results are registered as mean±standard deviation (SD) with statistical significance set at 5%.

RESULTS

According to the Table 1, MS patients with the mean age of 32.4±7.5 were found to be age matched with healthy control group with that of 31.7 ± 6.9. The participants were also sex matched and of the 50 MS patients, 11 (22%) and 39 (78%) individuals were male and female respectively; healthy subjects were built of 13 (26%) males and 37 (74%) females. The expanded disability status scale score (EDSS) of the RRMS patients was 2.5±1.16; the score was 2.3±1.32 and 2.7±1.03 for the males and females, respectively. The disease duration of the patients was 4.8 ± 2.1 in total, which was 4.5±2.3 and 5.2±1.8 for the males and females, respectively.

Table 1. Demographic specifications of RRMS patients and healthy control group

Subjects	N (%)	Age	Disease Duration	EDSS
HS	50	31.7±6.9	-	-
Male	13 (26%)	30.1±4.8	-	-
Female	37 (74%)	33.4±8.2	-	-
MS	50	32.4±7.5	4.8 ± 2.1	2.5±1.16
Male	11 (22%)	34.5±5.4	4.5 ± 2.3	2.3±1.32
Female	39 (78%)	30.3±5.6	5.2 ±1.8	2.7±1.03

EDSS: Expanded Disability Status Scale; HS: Healthy Subjects; MS: Multiple Sclerosis

Table 2. Gene expression fold change of PD-1, PD-L1, and PD-L2 mRNA in PBMCs from RRMS patients in comparison to healthy subjects

Target Gene	Fold Change (MS *vs.* HS)	*p*-value
PD-1	-2.91	*p*=0.003
PD-L1	-3.01	*p*=0.012
PD-L2	-1.24	*p*=0.067

PD-1: Programmed Cell Deth-1; PD-L1, 2: Programmed Cell Death Ligand 1, 2; MS: Multiple Sclerosis; HS: Healthy Subjects
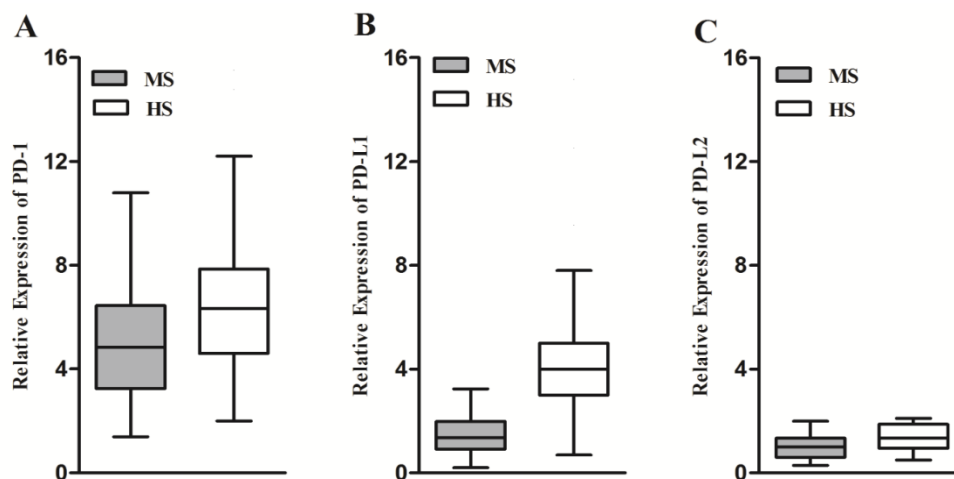

Figure 1. Relative expression of PD-1 (A), PD-L1 (B), and PD-L2 (C) mRNA in RRMS patients and the healthy subjects is illustrated through box and whisker graphs. MS: Multiple sclerosis, HS: Healthy subjects

A significant difference was observed in the expression level of PD-1 between MS patients and healthy controls (Table 2, Figure 1.A). MS patients showed a downregulated (Fold change=- 2.91; *p*=0.003) mRNA expression of PD-1 compared with healthy controls. PBMCs from MS patients also expressed the PD-L1 mRNA at lower levels (Fold change= -3.01; *p*=0.012) in comparison to healthy subjects (Table 2, Figure 1.B). However, the difference in the expression level of PD-L2 (Table 2, Figure 1.C) was not significant between MS patients and healthy individuals (Fold Change=-1.24; *p*=0.067).

Relative expression level of PD-1 did not correlate with age (*p*=0.513, *r*=−0.132) and disease duration (*p*=0.621, *r*=0.192) of RRMS patients. However, the expression level of PD-1was found to be negatively correlated (*p*=0.008, *r*=−0.763) with the EDSS score of the patients (Table 3, Figure 2). None of the age (*p*=0.324, *r*=0.181), disease duration (*p*=0.412, *r*=−0.133), and EDSS score (*p*=0.766, *r*=0.174) of RRMS patients correlated with relative expression level of PD-L1 (Table 3, Figure 3).

Table 3. Correlations of the relative expressions of PD-1 and PD-L1 mRNAs in PBMCs from RRMS patients with age, disease duration, and EDSS score

Parameter	PD-1		PD-L1	
	r	*p* value	r	*p*-value
Age	0.132	0.513	0.181	0.324
Disease Duration	0.192	0.621	-0.133	0.412
EDSS	-0.763	0.008	0.174	0.766

EDSS: Expanded Disability Status Scale; PD-1: Programmed Cell Deth-1; PD-L1: Programmed Cell Death Ligand 1; r: Pearson's Correlation Coefficient
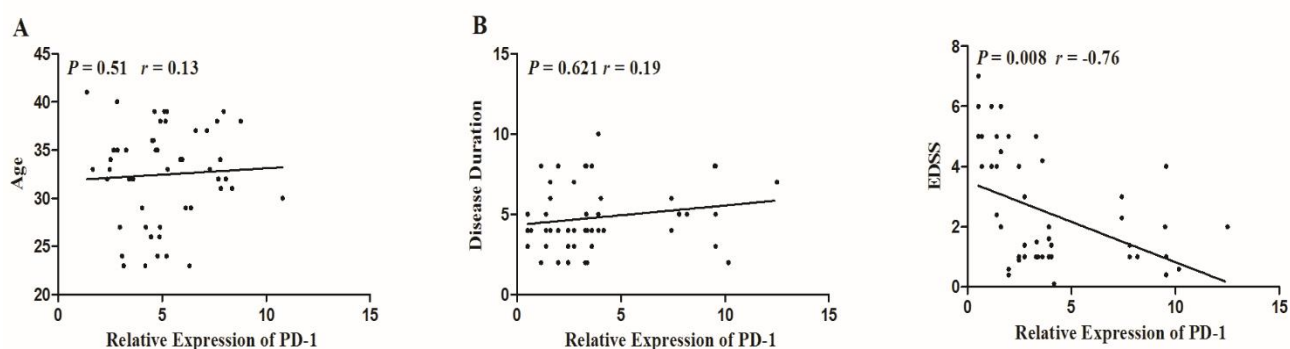
Figure 2. Scatter plots depict correlations of the relative expression of PD-1 in RRMS patients with the age (A), Disease duration (B), and EDSS score (C) (MS: Multiple sclerosis, HS: Healthy subjects, EDSS: Expanded disability status scale)

DISCUSSION

Co-inhibitory molecules of the B7-CD28 family are important players in the control of T-cell immunity. Inhibitory signals by the powerful B7-H1-PD-1 pathway are of crucial relevance for the mechanisms maintaining tolerance and terminating immune response in peripheral tissues to limit the organ damage.[20] Subsequent studies have demonstrated that PD-1/PD-L1 interaction, and not PD-1/PD-L2, can regulate the severity of Experimental Autoimmune Encephalomyelitis (EAE) which is considered as a mouse model for multiple sclerosis. Moreover, the loss of PD-L1 has been reported to cause onset of this pathology even in an EAE-resistant mouse strain.[21] Association between PDCD1 polymorphisms and several autoimmune diseases, including systemic lupus erythematosus, type 1 diabetes, rheumatoid arthritis, ankylosing spondylitis, men autoimmune infertility and multiple sclerosis, also suggests a key function for this pathway in the pathogenesis of human autoimmune diseases.[22,23]

In an animal model, Carter et al. showed that PD-1$^{-/-}$ and PD-L1$^{-/-}$ mice immunized with MOG[35-55], develop more severe EAE compared to wild type controls. This experiments on PD-1$^{-/-}$ mice are consistent with the antibody studies and confirm a role for PD-1 in EAE.[21]

Our study provides evidence that imbalanced expression of PD-1 and PD-L1 gene is associated with disease progression in MS patients. The mRNA expression level of both PD-1 and PD-L1 was found to be down-regulated in RRMS patients (Table 2, Figure 1). The PBMCs from patients showed significant lower expression of PD-1 (fold change=-2.91) and PD-L1 (fold change=-3.01). However, RRMS patients expressed PD-L2 mRNA in the PBMCs almost at the similar level to the healthy control group (fold change=-1.24). Higher expression of this immunosuppressive molecule during remission (of MS patients in this study) could be attributed to the natural mechanism by which the immune system forces itself back into lower response in order to avoid autoimmunity.

Autoantibodies to PD-L1 have been found in patients with rheumatoid arthritis and correlate with active disease[24] which suggest that blockade of the inhibitory function of PD-1/PD-L through autoantibodies may contribute to the development of autoimmune disease. Furthermore, Schreiner et al. demonstrated that PD-L1 mRNA transcripts in IFN-β treated multiple sclerosis had been increased eight-fold more than before treatment[25] which suggest that PD-1/PD-L pathway limits the damage caused by overaggressive T cells. In this study, the expression level of PD-1 and PD-L1 was significantly downregulated in RRMS patients compared with the healthy individuals. Moreover, decreased expression level of PD-1 was correlated significantly (p=0.008, r=−0.763; Table3, Figure.2) with increased EDSS score of the patients. Although insignificant, the relative expression of PD-L1 tended to negatively correlate with the disease duration (Table 3, Figure 3). It can be prematurely implied, seemingly, that lower expression of PD-1 might lead to worsening the disability manifestation of RRMS patients by means of over-activated immune cells, especially T cells.
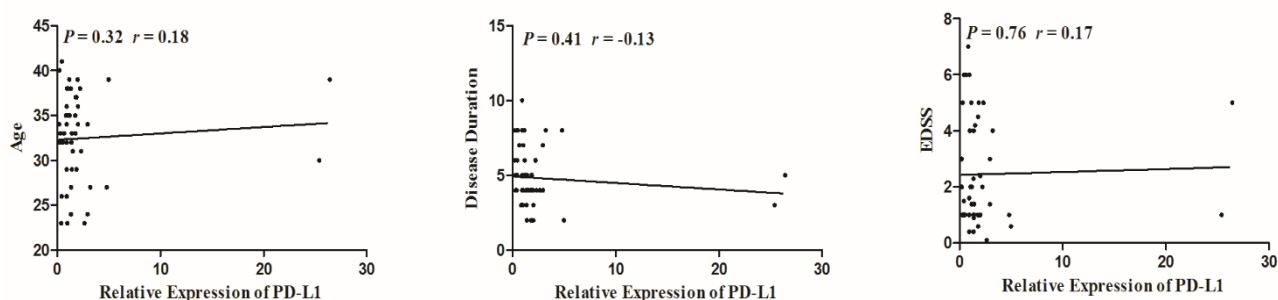
Figure 3. Scatter plots depict the correlations of the relative expression of PD-L1 in RRMS patients with the age (A), Disease Duration (B), and EDSS score (C) (MS: Multiple Sclerosis, HS: Healthy Subjects, EDSS: Expanded Disability Status Scale)

All in all, it was observed that there was an aberrant expression profile of immunosuppressive molecules in PBMCs of MS patients using Real-Time PCR method. To the best of our knowledge, this is the first study carried out over PD-1, PD-L1, and PD-L2 expression status in MS patients. Our results were mostly consistent with the previous studies on immune cells located in the inflammatory sites of autoimmune patients. The disturbance of peripheral tolerance affecting the inhibitory co-stimulatory pathways of T cells is a key element for the development of autoimmune disorders. These data are likely to improve our understanding of the crosstalk between the immune and the nervous system with special respect to the role of PD-1 and PD-L1 contributing to the perpetuation of MS disease and also can provide a clue for further studies in order to investigate the importance of PD-1 as a marker of disease activation/remission.

ACKNOWLEDGEMENTS

REFERENCES

1. Tobón GJ, Pers J-O, Cañas CA, Rojas-Villarraga A, Youinou P, Anaya J-M. Are autoimmune diseases predictable? Autoimmun Rev 2012; 11(4):259-66.
2. Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. J Exp Med 2000; 192(7):1027-34.
3. Latchman Y, Wood CR, Chernova T, Chaudhary D, Borde M, Chernova I, et al. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. Nat Immunol 2001; 2(3):261-8.
4. Chen L, Flies DB. Molecular mechanisms of T cell co-stimulation and co-inhibition. Nat Rev Immunol 2013; 13(4):227-42.
5. Gianchecchi E, Delfino DV, Fierabracci A. Recent insights into the role of the PD-1/PD-L1 pathway in immunological tolerance and autoimmunity. Autoimmun Rev 2013; 12(11):1091-100.
6. Goldenberg MM. Multiple sclerosis review. P T 2012; 37(3):175-84.
7. Simpson S, Blizzard L, Otahal P, Van der Mei I, Taylor B. Latitude is significantly associated with the prevalence of multiple sclerosis: a meta-analysis. J Neurol Neurosurg Psychiatry 2011; 82(10):1132-41.
8. Miller D, Barkhof F, Montalban X, Thompson A, Filippi M. Clinically isolated syndromes suggestive of multiple sclerosis, part I: natural history, pathogenesis, diagnosis, and prognosis. The Lancet Neurol 2005; 4(5):281-8.
9. Hemmer B, Kerschensteiner M, Korn T. Role of the innate and adaptive immune responses in the course of multiple sclerosis. Lancet Neurol 2015; 14(4):406-19.
10. Khondkarian O, Zavalishin I, Nevskaia O. [Classification of multiple sclerosis]. Zh Nevropatol Psikhiatr Im S S Korsakova 1983; 83(2):164-6.
11. Noseworthy JH. Progress in determining the causes and treatment of multiple sclerosis. Nature 1999; 399 (6738 Suppl):A40-7.
12. Compston, A. and A. Coles, Multiple sclerosis. Lancet 2002; 359 (9313):1221-31.
13. Anaya J-M. The autoimmune tautology. Arthritis Res Ther 2010; 12(6):147.
14. Jafari N, Shaghaghi H, Mahmoodi D, Shirzad Z, Alibeiki F, Bohlooli S, et al. Overexpression of microRNA biogenesis machinery: Drosha, DGCR8 and Dicer in

multiple sclerosis patients. J Clin Neurosci 2015; 22(1):200-3.

15. Kroner A, Mehling M, Hemmer B, Rieckmann P, Toyka KV, Mäurer M, et al. A PD-1 polymorphism is associated with disease progression in multiple sclerosis. Ann Neurol 2005; 58(1):50-7.

16. Ortler S, Leder C, Mittelbronn M, Zozulya AL, Knolle PA, Chen L, et al. B7-H1 restricts neuroantigen-specific T cell responses and confines inflammatory CNS damage: Implications for the lesion pathogenesis of multiple sclerosis. Eur J Immunol 2008; 38(6):1734-44.

17. McDonald WI, Compston A, Edan G, Goodkin D, Hartung HP, Lublin FD, et al. Recommended diagnostic criteria for multiple sclerosis: guidelines from the International Panel on the diagnosis of multiple sclerosis. Ann Neurol 2001; 50(1):121-7.

18. Polman CH, Reingold SC, Banwell B, Clanet M, Cohen JA, Filippi M, et al. Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. Ann Neurol 2011; 69(2):292-302.

19. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. Nat Protoc 2008; 3(6):1101-8.

20. Chen L. Co-inhibitory molecules of the B7-CD28 family in the control of T-cell immunity. Nat Rev Immunol 2004; 4(5):336-47.

21. Carter LL, Leach MW, Azoitei ML, Cui J, Pelker JW, Jussif J, et al. PD-1/PD-L1, but not PD-1/PD-L2, interactions regulate the severity of experimental autoimmune encephalomyelitis. J Neuroimmunol 2007; 182(1-2):124-34.

22. Sharpe AH, Wherry EJ, Ahmed R, Freeman GJ. The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection. Nat Immunol 2007; 8(3):239-45.

23. Zamani MR, Asbagh FA, Massoud AH, Salmaninejad A, Massoud A, Rezaei N. Association between a PD-1 gene polymorphism and antisperm antibody-related infertility in Iranian men. J Assist Reprod Genet 2015; 32(1):103-6.

24. Dong H, Strome SE, Matteson EL, Moder KG, Flies DB, Zhu G, et al. Costimulating aberrant T cell responses by B7-H1 autoantibodies in rheumatoid arthritis. J Clin Invest 2003; 111(3):363-70.

25. Schreiner B, Mitsdoerffer M, Kieseier BC, Chen L, Hartung HP, Weller M, et al. Interferon-beta enhances monocyte and dendritic cell expression of B7-H1 (PD-L1), a strong inhibitor of autologous T-cell activation: relevance for the immune modulatory effect in multiple sclerosis. J Neuroimmunol 2004; 155(1-2):172-82.