Toll Like Receptor 2 and 4 Expression in Peripheral Blood Mononuclear Cells of Multiple Sclerosis Patients

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ABSTRACT

Background: Multiple sclerosis (MS) is a T cell mediated autoimmune disease with unknown etiology. Appropriate MS therapeutic strategies need thorough understanding of both disease etiology and pathogenesis mechanisms. Ligation of TLR-2 and TLR-4 stimulates the production of several cytokines leading to CNS autoimmunity and neurodegenerative diseases. **Objective:** To find a relationship between MS disability and TLR-2 and TLR-4 expression on mononuclear cells in the blood of MS patients. **Methods:** Forty-five new case (NC) MS patients (33 females and 12 males) and 45 age and gender-matched healthy controls (HC) were recruited to the study. PBMCs were prepared and the expressions of TLR-2 and TLR-4 were assessed by flowcytometry technique using appropriate monoclonal antibodies. **Results:** Our results showed that the expression of TLR-2 and TLR-4 proteins in the patients group was significantly higher than that of healthy controls. TLR-2 but not TLR-4 was correlated with expanded disability status scale (EDSS) scores. **Conclusion:** High expressions of TLR-2 and TLR-4 may represent a state of innate immune activation in patients with MS.

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INTRODUCTION

Multiple Sclerosis (MS), a chronic inflammatory demyelinating disease of the central nervous system, is the major cause of neurological disability which mostly affects young adults (1). There are four forms of the disease the most common form of which is Relapsing-Remitting Multiple Sclerosis (RRMS), presenting with neurological dysfunction followed by phases of stabilization or partial to absolute healing. The next form is Secondary Progressive Multiple Sclerosis (SPMS), which expand in patients that already present with RRMS, is recognized by the severity of disability. The third form is Primary Progressive Multiple Sclerosis (PPMS), in which the first (primary) symptoms and disability progressively worsen over the time after illness inception. The last form is Progressive-relapsing MS (PRMS) which is progressive from the begining, with intermittent flare-ups of worsening symptoms without periods of remission (2). The Expanded Disability Status Scale (EDSS) is a method for measuring disability in MS and monitoring changes in the intensity of disability over time. The numeric range of EDSS is between 0-10 (3).

It is broadly believed that MS is an autoimmune disease (4-6). There are three important pieces of evidence supporting that MS is an autoimmune disease. First, specific T cells against myelin antigen can be isolated from peripheral blood mononuclear cells (PBMC) of individuals with the disease (7). Second evidence supports the existence of altered peptide ligands (APL) in humans which have partial agonistic or antagonistic properties in interaction with autoreactive T cells. The next evidence is Experimental Autoimmune Encephalomyelitis (EAE), a demyelinating disease that mimics MS in many aspects and expands neurological signs and symptoms in animal models (8).

In addition to the above mentioned features, similarity of MS with other autoimmune diseases, association with HLA genes (4), associations with other autoimmune disorders (9), and the presence of autoreactive B and T lymphocytes increase in the CNS provide more support to the autoimmune basis of MS (10), and can be a basis for therapeutic effects of corticosteroids (13) and efficacy of plasmapheresis (12).

Maturation of antigen presenting cells (APCs) expression of Toll-like receptors (TLR) (13) and activation of T cells and B cells via surface or intracellular receptors play important roles in the activity of the immune system. It is shown that immune cells such as monocytes, dendritic cells (DCs), B lymphocytes, T helper and T cytotoxic cells migrate to the CNS and mediate myelin destruction, axon damage, neuroinflammation and neurodegeneration (14,15). Both resident and infiltrated immune cells of the CNS express TLRs and expression of some types of TLR molecules increases in MS. The elevated TLR expression in the CNS may play a role in the pathogenesis of the disease (16,17).

TLRs mediate responses to self components such as ssRNA, dsRNA, CpG-DNA, high mobility group box 1 (HMGB1), HSP70, HSP90 and Pathogen Associated Molecular Patterns (PAMPs). TLR-ligand binding can trigger innate immune responses such as inflammation, initiate leukocyte migration and also prime adaptive immune responses (18-20) against particular microbial agents through molecular mimicry to self antigens and cross-reactivity. These processes are know to have the potential in triggering autoimmune process (21).

Ligation of TLR-2 and TLR-4 stimulates the production of IL-1, IL-6 and IL-12. These cytokines induce the differentiation of naive T cells into Th1 and Th17 lymphocytes. Th17 and Th1 lymphocytes produce IL-17 and IFN-γ, respectively. IL-17/IFN-γ-

producing lymphocytes ease leukocyte migration across the blood-brain barrier and play a role in CNS damage. In addition, IL-1 and IL-6 suppress the differentiation of induced regulatory T cells (iTregs). Tregs are a main source of IL-10, the cytokine which plays an important role in inhibiting CNS autoimmunity (22). Ligation of TLR-2 or TLR-4 on myeloid dendritic cells (DCs) can also stimulate the production of IL-23, that promotes the production of IL-17A by CD4 T cells (23). TLR-4 ligation also supports the production of IL-12 p70. This cytokine induces IFN-γ production, which induces cellular immunity (24). It is therefore logical to assume that TLR-2 and TLR-4 ligation are able to contribute in CNS autoimmunity.

The aim of present study was to investigate the relation between neuronal injury with TLR-2 and TLR-4 expression of mononuclear cells in the blood of patients with MS.

MATERIALS AND METHODS

This study was approved by the Ethical Committee on Human Research, Shahre Kord University of Medical Sciences. The volunteers were refferred to MS Clinic of Kashani University Hospital in Isfahan, Iran by clinician. The participants were informed about the procedures in the study and those who were willing to participate, donated blood samples and signed the informed consent.

Table 1. Comparison of demographic characteristics and TLRs expression in MS patients and healthy controls.

	Groups				
	MS (n=45)			Control (n=45)	P Value
	RRMS (n=39)	PPMS (n=1)	SPMS (n=5)		
Age (year)	26.56 ± 13.58	17	48.2 ± 5.76	33.28 ± 9.92	0.45
Gender (Male/Female)	10/29	0/1	2/3	12/33	0.75
TLR-2	11 (3.9-16.1)			3.6 (2.3-7.9)	0.002
TLR-4	14.5 (6.2-20.5)			2.2 (1.0-3.8)	<0.0001

● Data are Mean±SD, Median (IQR) and N (%)
●P Values derived from *t-test*, Chi-square and Mann-Whitney

Study Groups. Fourthly-five new cases (NC) of MS [33 females and 12 males, and 45 age and gender-matched healthy control (HC) individuals were recruited into the study. Characteristics of patients including type of MS and EDSS scores were evaluated by an experienced neurologist. Accordingly, 39 patients were categorized as RRMS, 1 PPMS and 5 SPMS.

All blood samples were collected before each drug administration. Patients were clinically stable with an age ranging between 17-58 years (mean 32.2 ± 10.6) years diagnosed with MS according to the McDonald diagnostic criteria with EDSS between 0-3 (Table 1) (25).

Isolation and Evaluation of Peripheral Blood Mononuclear Cells E. Ten milliliters of venous blood was obtained from each subject. PBMCs were isolated using Ficoll-Hypaque density gradient (Lymphodex, Germany). PBMCs were washed three times with Phosphate Buffer Solution (PBS) and the pellet was suspended in a solution composed of 92% fetal calf serum (Sigma, Germany) plus 8% DMSO (Sigma, Germany) and stored in liquid nitrogen. Before use, the cells were rapidly thawed, washed and resuspended in 4 ml of RPMI 1640 medium (Gibco, Germany). The cell number was estimated using light microscopy and the viability of the cells was checked by trypan blue (0.4%).
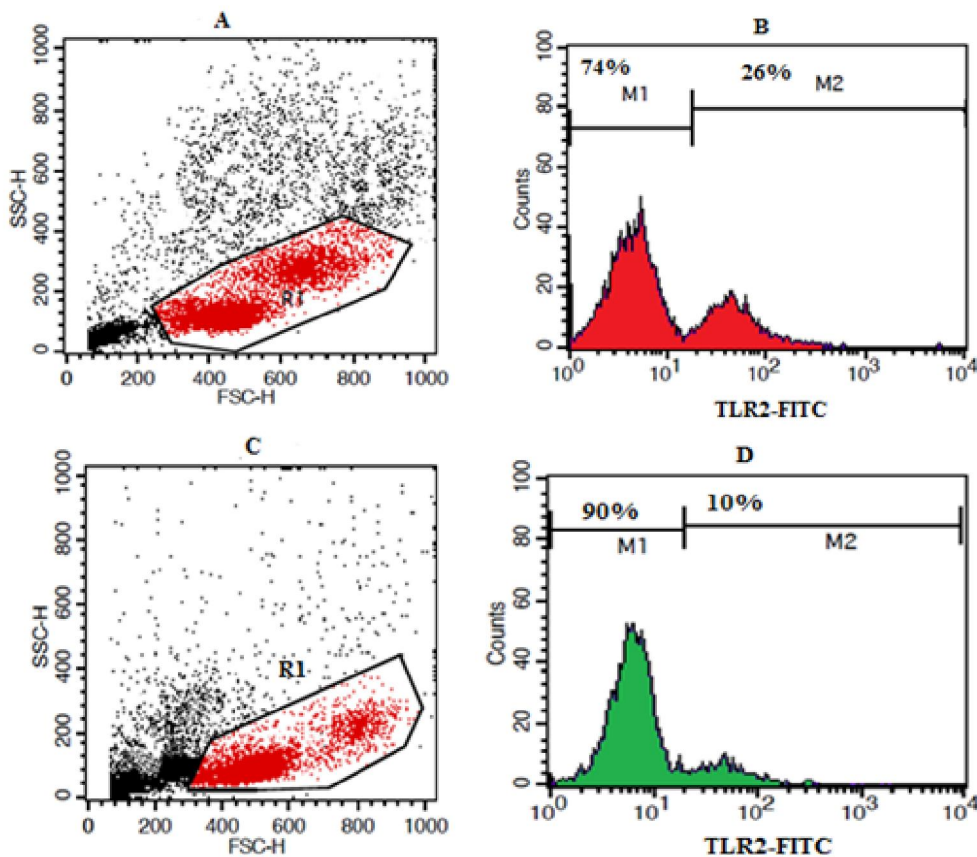

Figure 1. Representative Forward Scatter/Side Scatter (FSC/SCC) plot of PBMC by flow cytometry in patient (A) and control sample (C). Flow cytometric histograms of TLR2 expression on PBMC in MS patient (B) and healthy control (D). (Isotype control: M1, TLR2-Positive cells: M2).

Flowcytometry for Expression of TLR-2 and TLR-4. To determine the TLR-2 and TLR-4 surface expression, PBMCs were stained using the following monoclonal

antibodies: FITC-conjugated TLR-2 (ab13553, Abcam, UK) and FITC-conjugated antibodies TLR-4 (ab45126, Abcam, UK) and monoclonal antibody (IgG) FITC (Dako, X0932, USA) as an isotype control according to the manufacturer's instructions. Briefly, 100 µl of the cells were incubated with 2 µl of anti-TLR antibodies and isotype control for 30 min at 4ºC. Then the cells were washed twice and resuspended in 500 µl PBS, containing 0.5% formaldehyde. Samples were analyzed using BD FACS Calibur flow cytometer using BD Cell Quest software (Figures 1 and 2).
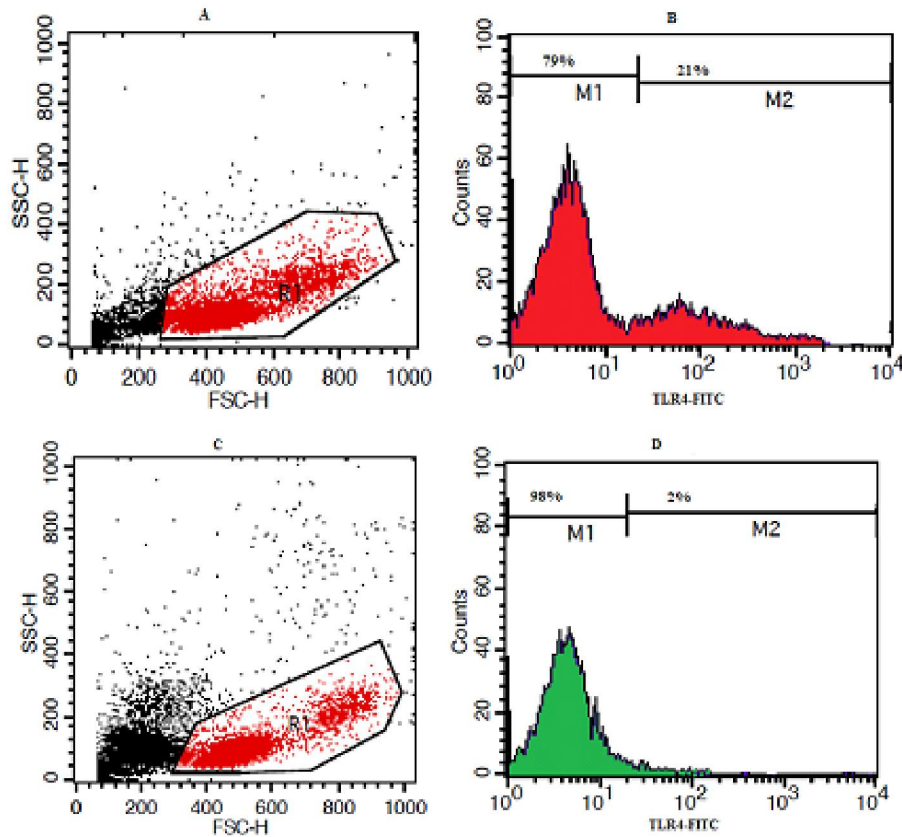

Figure 2. Representative Forward Scatter/Side Scatter (FSC/SCC) plot of PBMC by flow cytometry in patient (A) and control sample(C). Flow cytometric histograms of TLR4 expression on PBMC in MS patient (B) and healthy control (D). (Isotype control: M1, TLR4-Positive cells: M2).

Statistical Analysis. The data presented as Mean ± SD and Median (25th and 75th percentile) for continuous variables and number (percent) for categorical ones. Because the number of patients in each group was smaller than 50, we used the Shapiro-Wilk test for normality. Statistical differences among studied groups were assessed by Independent Samples *t-test*, Mann-Whitney, Pearson Chi-square and Pearson correlation test. All analyses were done using Statistical Package for Social Sciences version 20 (SPSS Inc., Chicago, IL, USA) and P-values less than 0.05 were considered statistically significant.

RESULTS

Ninety participants (66 men and 24 women) entered to the study. The ages ranged from 17 to 58 years, with a mean age of 32.15 ± 10.69 years and a median age of 32 years. Other demographic and clinical features of the study population are shown in detail in Table 1. As shown in Table 1, the age and sex structure of the studied groups is well distributed (p>0.05).
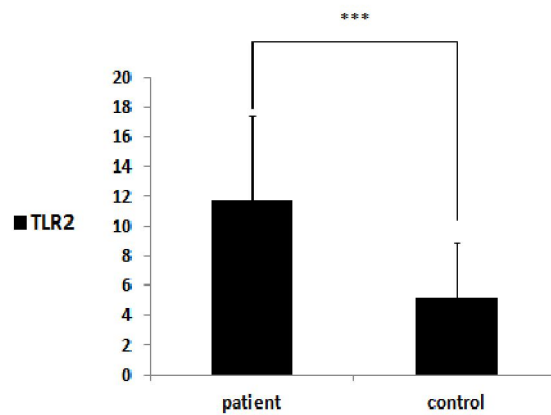

Figure 3. TLR-2 expression on PBMCs from MS patients and healthy controls. The columns of graphs showed the total results, and values were expressed as Mean Fluorescence Intensity (MFI). The expression of TLR-2 on PBMCs was significantly higher in new case patients compared to the healthy controls (p=0.0004).

TLR-2 and TLR-4 expression on Mononuclear Cells Surface. The expression of TLR-2 on the surface of mononuclear cells in new case MS patients and healthy donors was quantified using flowcytometry analysis. Cell surface expression of TLR-2 in new case patients and healthy donors was 11.79 ± 5.70 and 5.18 ± 3.73, respectively, which was significantly different (p=0.0004). Mean fluorescence Intensity (MFI) of TLR-2 in new case patients was also significantly higher than healthy donors (p=0.0004, Figure 3).
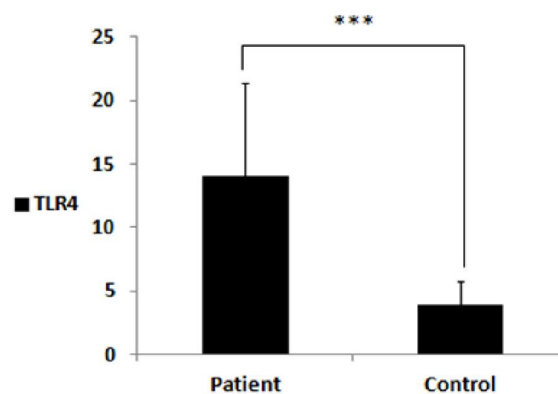

Figure 4. TLR-4 expression on PBMCs from MS patients and healthy controls. The columns of graphs showed the total results, and values were expressed as Mean Fluorescence Intensity (MFI). The expression TLR-4 on PBMCs were significantly higher in new case patients group compared to the healthy controls (p=0.0001).

TLR-4 cell surface expression was also analyzed using flowcytometry. Cell surface expression of TLR-4 in new case patients and healthy donors was 14.09 ± 7.35 and 3.88 ± 1, respectively, which was significantly different (p=0.0001). The Mean fluorescence Intensity (MFI) of TLR-4 in new case patients was also significantly higher than healthy donors (p=0.0001, Figure 4).

Correlation of TLR-2 but not TLR-4 with EDSS Score. As shown in Figure 5, a statistically significant correlation was found between TLR-2 levels and EDSS scores (p=0.049, r=0.168). However, there was no significant correlation between TLR-4 levels and EDSS scores (p=0.58, r=0.09).
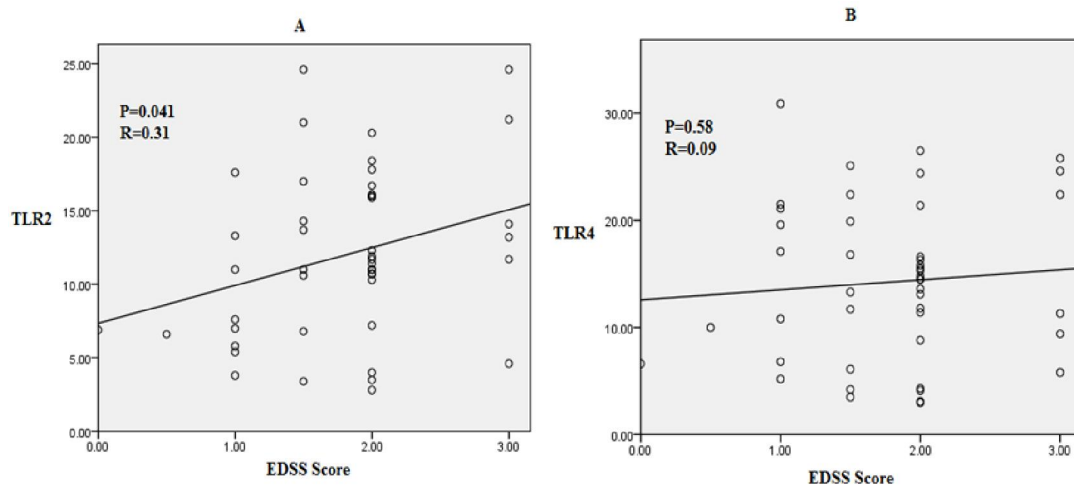

Figure 5. Correlation between TLR-2 and TLR-4 expression on PBMC and EDSS score in MS patients. A significant correlation between TLR2 and EDSS score is apparent (A). There is no significant correlation between TLR-4 and EDSS score (B).(Pearson correlation test).

DISCUSSION

The most suggested hypothesis about pathogenesis of MS disease and its phatophysiology is the autoimmune mechanism. Both innate and adaptive immunity are likely to take part in MS pathogenesis. It is now known that the innate immune responses, through the production of proinflammatory cytokines and also through antigen presentation play a very important role in the activation of myelin-specific autoreactive T cells. Therefore, the innate and adaptive immune systems cooperate in MS and EAE pathogenesis. It is believed that Monocytes/Macrophages and Th1/Th17 cell subsets contribute in this process (26). These cells express TLRs and recognize PAMPs on the surface of pathogens. Following PAMPs binding to TLRs, innate immune cells produce proinflammatory cytokines and act as APCs to prime naive T cells for recognizing antigens (27).

Our study showed that TLR-2 and TLR-4 on PBMCs correlate with MS neurodegeneration. We have identified TLR-2 and TLR-4 overexpression on PBMC of MS patients in comparison with healthy controls. Bystander activation and molecular mimicry are the suggested mechanisms for triggering autoimmune responses in the CNS by pathogens, which are likely to be mediated by TLRs. Tissue damage can unmask myelin antigens, resulting in epitope spread and expansion of autoimmunity (28-29).

TLR-2 is a ligand for Gram-positive bacteria such as *Staphylococcus aureus* and *Streptococcus pneumoniae*. Cell wall of these bacteria contains molecules such as peptidoglycan (PGN), which act as PAMPs and can activate TLR-2. Some viral molecules can also activate immune system via TLR-2 or TLR-4 as seen in Herpes simplex virus 1 (30).

Our study shows the relation between expression of TLR-2, TLR-4, and neuronal damage in the CNS in NC patients with MS.

There is evidence showing the active role of TLR-2 in EAE pathogenesis. *S. aureus* PGN added to Incomplete Freund's Adjuvant (IFA) stimulates EAE development in C57BL/6 mice (13). Another study showed that there is an association between PGN and APCs in the CNS of MS patients as well as in non-human primates (31). These studies suggest that PGN and may be the other TLR agonists can access the CNS during EAE, which might ease the reactivation of myelin-reactive T cells in the CNS tissue during EAE and MS and pathogenesis proces. These studies also showed that the effects of *S. pneumoniae* on EAE were TLR-2 dependent, because animals with TLR-2 deficiency could not develop more severe EAE (15).

LPS is the only well known ligand for TLR-4. Other exogenous or endogenous TLR ligands may be important in pathogenesis of MS. TLR-4 can trigger signaling, both MyD88-dependent pathway and MyD88-independent pathways that can explain the somewhat paradoxical results of the effects of TLR-4 agonists in modulating EAE. Ligand binding to TLR-2 and TLR4 induces the production of cytokines such as IL-1, IL-6 and IL-12, which induce the differentiation of naive T lymphocytes into Th1 and Th17 cells. Th17 and Th1 cells produce IL-17 and IFN-γ. These cells ease leukocyte movement across the blood-brain barrier and can also attack CNS tissue (32-35).

In the present study the levels of TLR-2 and TLR-4 expression were measured to explore the possible relation between TLR-2 and TLR-4 expressions with autoimmune mechanism of neurodegeneration in MS disease. Our results showed that there are TLR-2 and TLR-4 overexpressions on PBMCs of patients with MS in comparison with healthy controls. The significant correlation of TLR-2 expression with EDSS suggested that this molecule is involved in pathological pathway of MS disabilities but the similar correlation was not found for TLR-4.

Therefore, TLR molecules may be used as the new targets for treatment of MS and may be introduced as MS screening biomarkers in suspicious and high risk groups such as relatives of patients for more investigations, disease activity monitoring, estimation of treatment effects and prognosis prediction. However, further studies are needed to support these claims.

ACKNOWLEDGEMENTS

REFERENCES

1   Compston A, Coles A. Multiple sclerosis. Lancet. 2008; 372:1502-17.
2   Goldenberg MM. Multiple sclerosis review. P T. 2012; 37:175-84.
3   Kurtzke JF. Clinical definition for multiple sclerosis treatment trials. Ann Neurol. 1994; 36:S73-9.
4   Tiwari JL, Hodge SE, Terasaki PI, Spence MA. HLA and the inheritance of multiple sclerosis: linkage analysis of 72 pedigrees. Am J Hum Genet. 1980; 32:103-11.
5   Barcellos LF, Kamdar BB, Ramsay PP, DeLoa C, Lincoln RR, Caillier S, et al. Clustering of autoimmune diseases in families with a high-risk for multiple sclerosis: a descriptive study. Lancet Neurol. 2006; 5:924-31.
6   Nakahara J, Maeda M, Aiso S, Suzuki N. Current concepts in multiple sclerosis: autoimmunity versus oligodendrogliopathy. Clin Rev Allergy Immunol. 2012; 42:26-34.
7   Pette M, Fujita K, Kitze B, Whitaker JN, Albert E, Kappos L, et al. Myelin basic protein-specific T lymphocyte lines from MS patients and healthy individuals. Neurology. 1990; 40:1770-6.
8   Gold R, Linington C, Lassmann H. Understanding pathogenesis and therapy of multiple sclerosis via animal models: 70 years of merits and culprits in experimental autoimmune encephalomyelitis research. Brain. 2006; 129:1953-71.
9   De Keyser J. Autoimmunity in multiple sclerosis. Neurology. 1988; 38:371-4.
10 Brennan KM, Galban-Horcajo F, Rinaldi S, O'Leary CP, Goodyear CS, Kalna G, et al. Lipid arrays identify myelin-derived lipids and lipid complexes as prominent targets for oligoclonal band antibodies in multiple sclerosis. J Neuroimmunol. 2011; 238:87-95.
11 Miller DM, Weinstock-Guttman B, Bethoux F, Lee JC, Beck G, Block V, et al. A meta-analysis of methylprednisolone in recovery from multiple sclerosis exacerbations. Mult Scler. 2000; 6:267-73.
12 Khatri BO. Experience with use of plasmapheresis in chronic progressive multiple sclerosis: the pros. Neurology. 1988; 38:50-2.
13 Visser L, Jan de Heer H, Boven LA, van Riel D, van Meurs M, Melief MJ, et al. Proinflammatory bacterial peptidoglycan as a cofactor for the development of central nervous system autoimmune disease. J Immunol. 2005; 174:808-16.
14 Deng C, Radu C, Diab A, Tsen MF, Hussain R, Cowdery JS, et al. IL-1 receptor-associated kinase 1 regulates susceptibility to organ-specific autoimmunity. J Immunol. 2003; 170:2833-42.
15 Waldner H, Collins M, Kuchroo VK. Activation of antigen-presenting cells by microbial products breaks self tolerance and induces autoimmune disease. J Clin Invest. 2004; 113:990-7.
16 Lafon M, Megret F, Lafage M, Prehaud C. The innate immune facet of brain: human neurons express TLR-3 and sense viral dsRNA. J Mol Neurosci. 2006; 29:185-94.
17 Zhou Y, Ye L, Wan Q, Zhou L, Wang X, Li J, et al. Activation of Toll-like receptors inhibits herpes simplex virus-1 infection of human neuronal cells. J Neurosci Res. 2009; 87:2916-25.
18 Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature. 2001; 413:732-8.
19 Wang T, Town T, Alexopoulou L, Anderson JF, Fikrig E, Flavell RA. Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis. Nat Med. 2004; 10:1366-73.
20 Tolouei S, Hejazi SH, Ghaedi K, Khamesipour A, Hasheminia SJ. TLR2 and TLR4 in Cutaneous Leishmaniasis Caused by Leishmania major. Scand J Immunol. 2013; 78:478-84.
21 Cirone M, Cuomo L, Zompetta C, Ruggieri S, Frati L, Faggioni A, et al. Human herpesvirus 6 and multiple sclerosis: a study of T cell cross-reactivity to viral and myelin basic protein antigens. J Med Virol. 2002; 68:268-72.
22 Miranda-Hernandez S, Baxter AG. Role of toll-like receptors in multiple sclerosis. Am J Clin Exp Immunol. 2013; 2:75-93.
23 Roses RE, Xu S, Xu M, Koldovsky U, Koski G, Czerniecki BJ. Differential production of IL-23 and IL-12 by myeloid-derived dendritic cells in response to TLR agonists. J Immunol. 2008; 181:5120-7.
24 Re F, Strominger JL. Toll-like receptor 2 (TLR2) and TLR4 differentially activate human dendritic cells. J Biol Chem. 2001; 276:37692-9.
25 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; 69:292-302.
26 Bettelli E, Korn T, Oukka M, Kuchroo VK. Induction and effector functions of T(H)17 cells. Nature. 2008; 453:1051-7.

27 Hacker H, Redecke V, Blagoev B, Kratchmarova I, Hsu LC, Wang GG, et al. Specificity in Toll-like receptor signalling through distinct effector functions of TRAF3 and TRAF6. Nature. 2006; 439:204-7.

28 Bailey SL, Schreiner B, McMahon EJ, Miller SD. CNS myeloid DCs presenting endogenous myelin peptides 'preferentially' polarize CD4+ T(H)-17 cells in relapsing EAE. Nat Immunol. 2007; 8:172-80.

29 McMahon EJ, Bailey SL, Castenada CV, Waldner H, Miller SD. Epitope spreading initiates in the CNS in two mouse models of multiple sclerosis. Nat Med. 2005; 11:335-9.

30 Villalba M, Hott M, Martin C, Aguila B, Valdivia S, Quezada C, et al. Herpes simplex virus type 1 induces simultaneous activation of Toll-like receptors 2 and 4 and expression of the endogenous ligand serum amyloid A in astrocytes. Med Microbiol Immunol. 2012; 201:371-9.

31 Visser L, Melief MJ, van Riel D, van Meurs M, Sick EA, Inamura S, et al. Phagocytes containing a disease-promoting Toll-like receptor/Nod ligand are present in the brain during demyelinating disease in primates. Am J Pathol. 2006; 169:1671-85.

32 Holmoy T. Immunopathogenesis of multiple sclerosis: concepts and controversies. Acta Neurol Scand Suppl. 2007; 187:39-45.

33 Aranami T, Yamamura T. Th17 Cells and autoimmune encephalomyelitis (EAE/MS). Allergol Int. 2008; 57:115-20.

34 Komiyama Y, Nakae S, Matsuki T, Nambu A, Ishigame H, Kakuta S, et al. IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. J Immunol. 2006; 177:566-73.

35 von Budingen HC, Menge T, Hauser SL, Genain CP. Restrictive and diversifying elements of the anti-myelin/oligodendrocyte glycoprotein antibody response in primate experimental allergic encephalomyelitis. Immunogenetics. 2006; 58:122-8.