Role of B Cells and Myelin Basic Protein-Induced Antibodies in the Pathogenesis of Experimental Autoimmune Encephalomyelitis in C57bl/6 J Mice

Sherman Maryam Sahlolbei[1,2], Maryam Hajizadeh[3], Marzieh Naseri[1,2], Leili Saeednejad Zanjani[2], Hossein Ghanbarian[4], Zahra Madjd[2], Seyed Mahmoud Hashemi[5,6] and Jafar Kiani[1,2*]

[1]Department of Molecular Medicine, Faculty of Advanced Technologies in Medicine, Iran University of Medical Sciences (IUMS), Tehran, Iran
[2]Oncopathology Research Center, Iran University of Medical Sciences (IUMS), Tehran, Iran
[3]Department of Parasitology and Mycology, Faculty of Medicine, Iran University of Medical Sciences (IUMS), Tehran, Iran
[4]Department of Medical Biotechnology, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[5]Department of Immunology, School of Medicine Shahid Beheshti University of Medical Sciences, Tehran, Iran
[6]Department of Tissue Engineering and Applied Cell Sciences, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

*Corresponding Author**
Jafar Kiani
Department of Molecular Medicine
Faculty of Advanced Technologies in Medicine
Iran University of Medical Sciences (IUMS),
Tehran, Iran
Email: ja.kiani@gmail.com

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Abstract

Background: Over the years, the treatment of Multiple Sclerosis (MS) has improved due to the dramatic advancement in immunology and neuroscience. The recent therapeutic approaches substantially expanded our view of the role of B cells in the progression of MS. The present study aimed to investigate the role of B cells and Myelin Basic Protein (MBP)-induced antibodies in developing Experimental Autoimmune Encephalomyelitis (EAE, an animal model of multiple sclerosis).

Method: We used MBP for induced experimental autoimmune encephalomyelitis in C57BL/6 female mice and analyzed the clinical score. The Enzyme-Linked Immunosorbent Assay (ELISA) and western blot assessed the expression of MBP-induced antibodies. The infiltration rate of the inflammatory cells and demyelination in the spinal cord were also evaluated by Hematoxylin/Eosin, Luxol fast blue, or anti-MBP staining procedures. Besides, the fluorescent Immunohistochemistry (IHC) was used to detect T and B cells infiltration in the spinal cord, and both T and B cells were counted by flow cytometry.

Result: These findings indicated that MBP immunization significantly increased B cells and anti-MBP-antibodies. IHC analysis revealed a significant increase in the penetration rate of immune cells and the development of nerve damage in the spinal cord. Moreover, MBP antibody levels directly correlate with immune cell infiltration in the spinal cord ($p< 0.001$).

Conclusion: This study supports the idea that reactive B cells and MBP-induced antibodies may contribute to the pathogenesis of EAE. Further studies are needed to investigate targeting B cells and MBP-induced antibodies as potential therapeutic strategies for treating multiple sclerosis

Keywords: Experimental autoimmune encephalomyelitis • Multiple sclerosis • B cells • Antibody • Neuro immunology • Myelin basic protein microglia

Introduction

Multiple Sclerosis (MS) is a chronic autoimmune inflammatory disease characterized by two major pathological hallmarks in the Central Nervous System (CNS), including the plaques or lesions composed of multiple focal areas of myelin loss and inflammation damage. The damage to nerve cells leads to a cascade of immune cell interactions and antibody production, resulting in a neurological disability in young adults [1-3]. Four main subgroups of MS, including Relapsing Remitting MS (RRMS), Primary Progressive MS (PPMS), Secondary Progressive MS (SPMS), and Progressive Relapsing MS (PRMS), show different pathogenetic patterns [4-6]. The progression of MS occurs in three steps; primarily, a pre-clinical stage, secondly, a Relapsing-Remitting MS (RRMS) clinical-stage known with discrete features of neurologic dysfunctions, such as optic neuritis, sensory disturbances, or disturbances in motor and cerebellar function, and in the end, the progressive clinical-stage which progressively worsened and neurologic dysfunctions, affecting specifically a patient's gait [7-10].

Neuroinflammation can be described as an inflammatory response within the CNS due to complex crosstalk between infiltrating immune cells and CNS-resident cells [11]. Although MS-related immune responses initiate as T-cell-mediated responses, according to the data regarding clinical trials, B cells and antibodies play a pivotal role in the initiation and propagation of MS and other autoimmune diseases[12-14]. Traditionally, the prime focus of the role of B cells in neuroinflammatory diseases was their ability to produce antibodies [15]. B lymphocytes play a decisive role both as Antigen-Presenting Cells (APCs) for the activation of T cells and as precursors of antibody-secreting plasma cells [16].Humoral immunity affects the inflammation and development of demyelinating lesions [17, 18]. Increased antibody levels in the spinal cord of MS patients were initially reported in 1942 by Kabat et al., who revealed that the self-reactive antibodies are involved in MS pathogenesis acting as an indicator in the diagnosis of this disease [19]. Molecular mimicry is popular theory about antibodies contends that T cells specific for CNS activation could be triggered by an infectious agent (herpes simplex virus, cytomegalovirus, Epstein-Barr and Rubella)or from the presentation of myelin antigens in the cervical lymph nodes [20]. Another theory of Grabar's "debris hypothesis" in autoimmunity is, During inflammation in an MS plaque, demyelination occurs, leading to damage to the multiple layers of the myelin nerve and making antibodies against the intracellular protein [21]. Brändle et al. provide evidence that Oligoclonal Bands (OCBs) in MS target ubiquitous intracellular antigens released in cellular debris [22].

Myelin Basic Protein (MBP) is one of the most abundant proteins in the human brain [23]. MBP is a prime example of a seemingly simple but

biochemically and structurally complex molecule. Closely linked to normal nervous system development and neurodegenerative disease [24]. MBP exists as several size and charge isoforms, some of which have a possible link to the pathogenesis of MS [25]. The role of pathogenic antibody confirmed in some diseases, but the mechanisms of MBP antibodies in pathogens MS remain largely unclear. Whether these antibodies actively contribute to the pathogenesis or progression of MS is still under debate [26].

As a result, more studies are necessary on the relationship between antibody levels and myelin damage during MS progression. B-cell therapy can affect the treatment of MS, and several B lymphocyte depletion therapies are available. Therefore, it is essentially more to understand the functions of B cells and antibodies to determine their role in the development and MS therapy. We aimed to explore further the role of B cells and MBP autoantibodies in pathogenesis MS and myelin damages. As well as, we investigate the relationship between antibody levels and the rate of immune cell infiltration of the central nervous system in mouse models.

Materials and Methods

Vector construction

pET28a/MBP-CFP plasmid (kanamycin-resistant; Novagen, USA) containing T7 promoter was used to express recombinant Myelin basic protein peptide fused to the Cyan Fluorescent Protein (rMBP-CFP). The recombinant plasmids were chemically transformed into the BL21 strain of *Escherichia coli* (*E. coli*) with $CaCl_2$. Firstly, the BL21 were grown in 15 ml of Luria-Bertani (LB) medium. After centrifuging, harvest pelleted bacterial cells were suspended gently in cold $CaCl_2$ (0.1 M) and incubated on ice for 1h. After centrifuging (2500 g /10 min), the competent bacteria were mixed with 1 µl of pET28a/ MBP-CFP plasmid and incubated on ice (30 min), placed at 42°C degrees for 45 seconds (heat shock), and then placed back in ice (2 min). In this approach, the transformed bacteria are incubated at 37°C for 30 min, then cultured on LB agar plates (contains 50 µg/ml kanamycin) incubated at 37°C for overnight. After selecting the colony, the plasmid isolated using the purification kit according to the manufacturer's protocol (Qiagen, mini, USA).

Protein expression and purification

For MBP expression, pET28a plasmids were transformed to *E. coli* BL21 (DE3) through chemical transformation. The BL21 were spread on LB agar plates containing kanamycin antibiotics and grown overnight at 37°C. A single colony was utilized to prepare overnight cultures in LB medium with the kanamycin antibiotics (50 µg/mL). The cultures were grown at 37°C with vigorous shaking until the bacteria density reached an optical density of 600 nm (OD600). The induction of protein (MBP) expression was performed at the end of the exponential bacterial growth phase using 0.5mM /mL Isopropyl β-D-1-Thiogalactopyranoside (IPTG) at 37°C for 6h. The BL21 were then harvested via centrifugation (2500 g /10 min), and the bacterial pellet was stored at -20 °C until further purification. Subsequently, the frozen bacterial pellets were lysed in lysis buffer (Lysozyme 10 µg/ml, PMCF 1 µg/ml) for three freezing and thawing cycles. The lysate was centrifuged (10000 g, 4°C, for 20 min) and the supernatant was removed. According to the manufacturer's protocol, the protein was isolated using a Nickel column (Ni–NTA kit, ABT, Spain). The protein extract was detected using UV280, which was confirmed by 15% SDS–PAGE and then concentrated by Amicon® Ultra Centrifugal Filter (Merck, Germany) and quantitated by the Bradford method(Kruger 1994).

Mice

The twenty-four female C57BL/6J mice (6 weeks-8 weeks old and with a weight range of 18 g-20 g) were obtained from the Pasteur Institute of Iran (Tehran, Iran). The animals were housed in a room under standard conditions at a temperature of 23°C ± 1°C. Four animals were kept in each cage (polypropylene cages; 42 cm ×27 cm ×15 cm), provided with unlimited access to food and water. They were kept on a 12-hour light-dark cycle and tested during the light phase. The mice were randomly selected for each experimental and control group. The experiments were conducted as blind to prevent any bias in the study results. According to the refined ARRIVE guidelines (Animal Research: Reporting *in vivo* Experiments), all experiments were performed [27].

Experimental autoimmune encephalomyelitis

For immunized C57BL/6J mice with MBP peptide using standard protocols and reagents. Briefly, EAE was induced in twelve female C57BL/6J mice by subcutaneous injection of rMBP-CFP (83-92) emolsion in Complete Freund's Adjuvant (CFA) [28-30]. Antigen concentration utilized for mouse immunization is generally around 50 µg per injection. Each mouse receives a 100 µl of emulsion subcutaneously. In parallel, twelve other female C57BL/6J mice were received the Phosphate-Buffered Saline (PBS) (control group). Followed by standard, pertussis toxin (Sigma) 400 ng in PBS was injected into the mouse flank. Subsequent immunizations were performed on day 14 (second booster) and 28 (Third booster) intervals with rMBP-CFP and complete Freund's adjuvant. The process was according to the experimental design summarized in Figure 1.

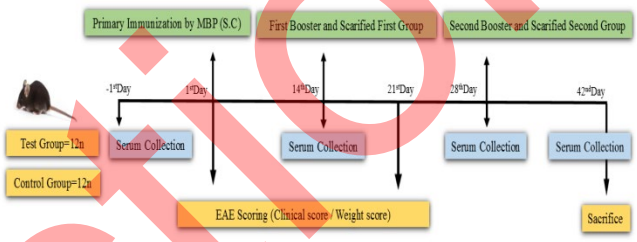


Figure 1. Overview of experimental design for the immunization experiments.

Following immunization, all the mice were examined daily for neurologic clinical signs according to the following criteria (0, healthy; 0.5, flaccidity and partial paralysis of the tail; 1, complete paralysis of the tail; 1.5, weakness in one hind limb; 2, weakness in both hind limbs; 2.5, partial hind limb paralysis; 3, complete hind limb paralysis; 3.5, partial forelimb paralysis; 4, complete forelimb paralysis; 5, mortality). Their body weights were measured according to the standard protocol. Bodyweight and the clinical score of all mice were registered every other day. At the end of each experiment, animals have immediately sacrificed with placed in the 100% carbon dioxide (CO_2) chamber for 2 min to 3 min. At this time point, it was ensured that the animals did not perceive pain during the procedure. To detect anti-rMBP-CFP antibody titer before immunization and on days 14, 28, and 42 post-immunization, the blood was taken from the mice's caudal vein then the serum was separated by centrifugation at 15,000 g for 30 min. To investigate the effect of increased antibody levels on spinal cord injury, we divided the animal into three groups: the first group was sacrificed on the 14th day following the immunization (4 immunized mice and 4 control mice), the second group on the 28th day (4 immunized mice and 4 control mice), and the third group on the 42nd day (4 immunized mice and control mice). With each test group, four control mice were sacrificed.

SDS–PAGE and Western blotting

The purified rMBP-CFP protein was analyzed utilizing Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (15% SDS-PAGE). To detect anti-MBP antibodies in blood from animals before and after immunization and were analyzed using western blot. Primarily, 1 µg of protein was loaded onto SDS-PAGE, after proteins were transferred onto a Polyvinylidene Fluoride (PVDF) membrane using the electrophoresis apparatus (Bio-Rad, Mini Trans-Blot, USA). Briefly, the PVDF membrane incubates in a blocking buffer (5% non-fat milk) for 30 minutes. Subsequently, the membrane was washed in the wash buffer TBST (Tris-buffered saline, 0.1% Tween 20 detergent) and incubated overnight with 1:100 dilution of polyclonal primary antibody (mouse serum). Afterward, it was incubated with a 1:1000 dilution of secondary antibody Goat anti-mouse IgG (Mab Tech, Sweden) and washed three times. Ultimately, the color was developed by incubating the membrane in a buffer containing chemiluminescence detection with Enhanced Chemiluminescence (ECL) (Promega, USA).

Histological and Immunohistochemical evaluation of spinal cords

After mice were sacrificed, their spinal cords were separated and perfused with 25 mL cold PBS. They were then fixed in 4% paraformaldehyde at 4˚C overnight. The tissues were paraffin-embedded and sectioned at 5 µm with the Developmental Biology Histology core at Firoozgar Hospital, Tehran, Iran. Sections that were 4 µm-6 µm thick were cut and stained.

The Luxol Fast Blue stain (LFB) was stained with Representative sections to detect myelin detrition. According to the standard protocol, Hematoxylin and Eosin staining (H & E) detect inflammatory cell infiltration [31]. Using a Nikon 90i motorized upright digital microscope, the slides were examined via optical microscopy and analyzed with ImageJ software. Fluorescent staining (Santa Cruz Biotechnology) was applied to observe T cells (CD3+) and B cells (CD45R+) infiltration in the spinal cord. The primary anti-CD3 (PC3/188A) with a concentration of 200 µg/ml was used for the detection of T cells (CD3+) of the mouse by immunofluorescence microscope (starting dilution 1:50, dilution range 1:50-1:500). Primary antibody against CD45R+ (HIS24-Sc19615) with a 200 µg/ml concentration was used to detect B-cell-specific CD45 isoform (dilution range: 1:50-1:200). Besides, the primary anti-MBP antibody (sc-271524) is used for detecting myelin. Following the addition of primary antibodies, FITC-conjugated goat anti-mouse IgG (sc-2010) with a concentration of 100 µg/ml was utilized (dilution range:1:100-1:400) as the secondary antibody. The stained samples were analyzed microscopically under fluorescent/FITC filter and ImageJ software. Histological analysis was repeated three times with technical replicates for each independent experiment.

ELISA

Blood samples were collected from the mice's tails before the immunization and on days 14, 28, and 42 post-immunization. Serum was obtained by centrifugation of the clotted blood at 15000 g for 30 min and was frozen at -20°C until used. The total serum MBP-specific IgG was quantified by serial dilutions in plate pre-coated with rMBP-CFP (with the concentration of 5 mg/ml) in the coating buffer 100 mM (sodium bicarbonate). The plates were blocked (5% non-fat dry milk containing 0.05% PBST) and incubated with sera overnight at 4°C. After being washed (PBS/Tween 0.5%). Add HRP-conjugated goat anti-mouse IgG (1:250), and the plates were incubated at room temperature for an additional 1 hour. The enzymatic reaction was stopped by adding 50 µl of 2N sulfuric acid. The optical density has measured the absorbance of ELISA at 450 nm against the reference wavelength of 630 nm using a plate reader.

Isolation of splenocytes from mouse spleen and Flow cytometry analysis

The spleens were removed from the immunized and control mice. Splenocytes were collected by flushing with 3 mL RPMI 1640. Red blood cells were lysed with lysis buffer and washed three times with complete medium (RPMI supplemented with 20 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin 10% FBS). The cells were incubated with antibodies (concentrated to 1'106 cells/ml). Two-color flow cytometry analysis (Bio Legend): FITC-Anti-Mouse CD45R (1.0 µg per million cells), PE- Anti-Mouse CD19 (0.25 µg per million cells) product for detecting B cell. The splenocytes (concentrated to 1'106 cells/ml) were incubated with PE-Anti-Mouse CD4 (0.25 µg per million cells) and APC-Anti-Mouse CD8 (0.25 µg per million cells) product for detecting T lymphocytes. Cell analysis was performed on a BD flow cytometer (BD FACSCalibur), and the results were analyzed using FlowJo software.

Statistical analysis

The statistical analyses were performed using SPSS, version 22.0 (Armonk, NY: IBM Corp). The Shapiro-Wilk's Test was used to determine the normal distribution. Pearson's or Spearman's correlation test (Bivariate analysis) was employed to analyze the significance of the correlation between clinical score and clinical data. The data were expressed as the mean (M); Standard Deviation (SD), and Median; quartile (Q1, Q3). The two-way ANOVA test was also used to compare clinical score severity in mice. Tukey's Multiple Comparison Test compares multiple groups in ELISA results. The p-value of less than 0.05 was considered statistically significant.

Results

Protein expression and purification

The expression of rMBP-CFP was induced with IPTG, as described. The cells were harvested, lysed, and processed for recombinant protein purification in two steps, first on a Ni column and then with Amicon® Ultra Centrifugal Filter. Sera from immunized mice and control were evaluated with western-blotting. The bands that matched the previously reported sizes of approximately 37 kDa for the MBP protein were detected. briefly illustrates the SDS-PAGE and the western blot analysis of the expressed rMBP(Figure 2 and Figure 3).

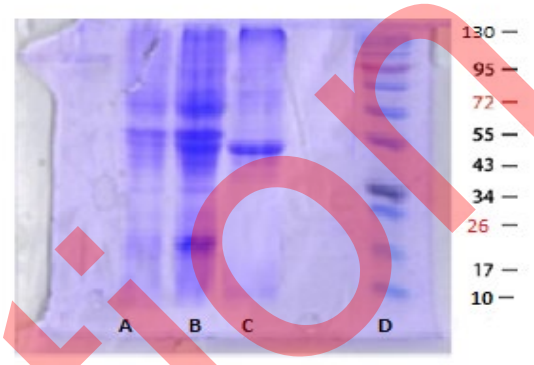


Figure 2. Results of protein electrophoresis SDS-PAGE. A) Bacterial lysate before induction with IPTG. B) Bacterial lysate after induction. C) Protein band after purification with a chromatography column. D) Protein marker.

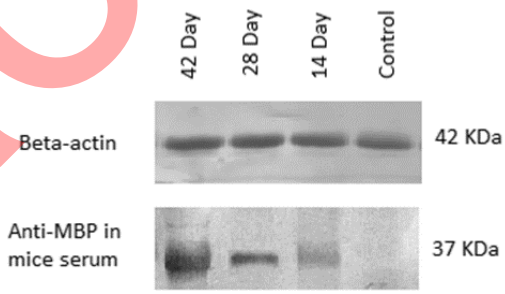


Figure 3. Western blot analysis of mice serum before and after rMBP injection. The Control group is the serum of mice before injection rMBP, 14th day, 28th day and 42nd day after immunization. Beta-actin was used to normalize the protein levels and confirm that protein loading is the same across the gel.

Neurological function and clinical score induced by rMBP-CFP immunization

The C57BL/6J mice were immunized using rMBP-CFP in three steps to investigate the clinical disease presentation. The neurological effects of the immunization with rMBP-CFP on the test group were evaluated daily until 21 days after the onset of the symptoms and compared to the control group (PBS). The onset of the clinical symptoms occurred between 11 to 14 days after the immunization. The day of disease manifestation and the severity of its symptoms in the test group were compared to those of the control group with the two-way ANOVA method. The Median (Q1, Q3 of clinical scores in the control group was 0 (Q1-Q3; 1-2), and in rMBP-CFP treated mice was 1 (Q1-Q3; 0-3). The statistical comparison with the two-way ANOVA method indicated a significant difference in rMBP-CFP-treated mice and control during the follow-up ($p < 0.001$) (Figure 4A).

The Median (Q1, Q3) weight in the PBS- and rMBP-CFP-received mice were 3.10 (Q1-Q3; 1-2) and 2.25 (Q1-Q3; 0.7- 2), respectively (Figure 4B).

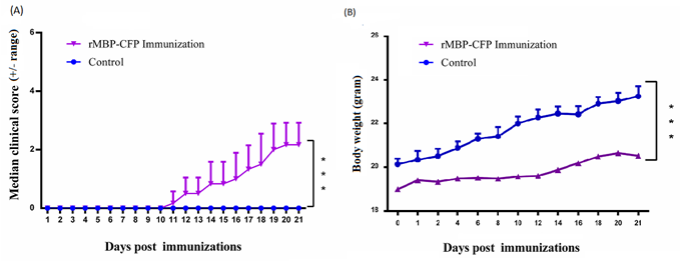


Figure 4. Scores were used for the evaluation of the symptoms of the immunization with rMBP-CFP (A) The clinical score in the mice immunized with rMBP post-immunization 0 (no signs) to 5 (complete paralysis). The Median clinical scores of the control and test groups were: 0 (Q1-Q3; 1-2) and 1(Q1-Q3; 0-3), respectively (B) The mice's Median body weights over the 3-week feeding study from the control and test groups were: 3.10 (Q1-Q3; 1-2) and 2.25 (Q1-Q3; 0.7- 2.1), respectively. (***p <0. 001).

Simultaneously with the increasing clinical score, experimental mice observed weight loss. We observed a correlation between this weight and the clinical score. In this study, bivariate analysis was performed to show the correlation between clinical score and weight. The bivariate analysis results revealed a statistically significant inverse correlation between clinical score and weight (Spearman's rho, p=0.049).

rMBP-CFP immunization and effect on humoral immune response in mice

ELISA assay of sensitivity and specificity for detecting and quantifying antibodies immunized with rMBP–CFP peptide. To determine the degrees of reactivity and specificity of the MBP antibody, the sera of the mouse from each group was detected by the ELISA technique. The IgG antibody kinetics and frequency were analyzed in sera obtained from the mice before the immunization and 14 days, 28 days, and 42 days after utilizing ELISA. As shown in Figure 5, the IgG antibodies in sera obtained from the immunized mice with rMBP-CFP protein showed an increase on days 14 (M=1.42, SD= 1.09), 28 (M=1.60, SD=1.0), and 42 (M=1.55, SD=1.0) compared to those before the immunization. The Pearson's correlation test exhibited a statistically significant difference between day 42 and before the immunization (p=0.034). Moreover, Tukey's Multiple Comparison Test elicited the statistically significant difference observed between day 42 and 14 days after immunization (p=0.009) and between day 28 and 14 days after immunization (p=0.041).

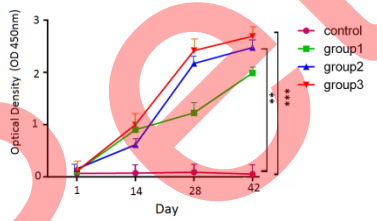


Figure 5. Humoral immune response to rMBP-CFP. ELISA analysis of antibodies against rMBP-CFP peptide in mice sera. Sera were collected from each mouse before the immunization and on the 14th, 28th, and 42nd days following the immunization. (**p< 0.01, ***p<0. 001).

rMBP-CFP induces or triggers neuropathology in immunization mice

The spinal cord is the early site of inflammatory damages in the murine system of EAE [32]. Hence, the mice's spinal cords were examined for pathologic changes. To evaluate the inflammation, H & E staining on the spinal cord of the animals was performed. The inflammatory cells, such as lymphocytes and neutrophils, are crucial in diagnosing inflammation. The localization and size of the inflammation were different in the three test groups; however, no statistically significant differences were observed between them (Figure 6A).

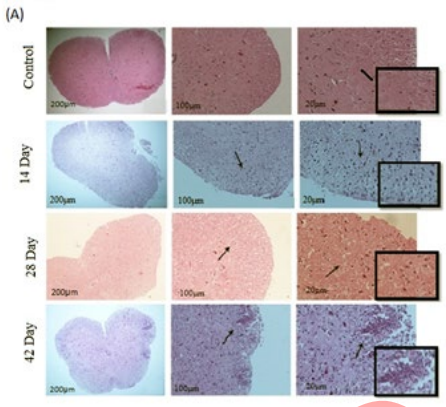


Figure 6A. H&E stained spinal cord sections of rMBP-CFP immunized mice exhibited signs of inflammation compared to the control group.

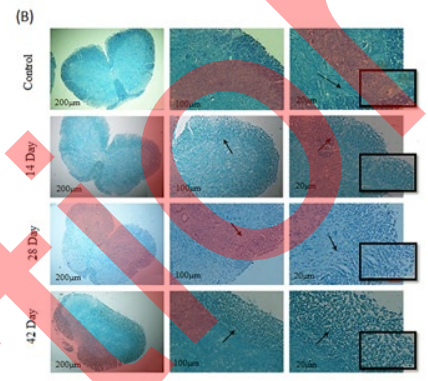


Figure 6B. Luxol Fast Blue staining in the spinal cord sections of rMBP-CFP immunized animals revealed demyelination indicated by white areas in the nerve. In the control nerves, the myelin sheaths were uniformly stained.

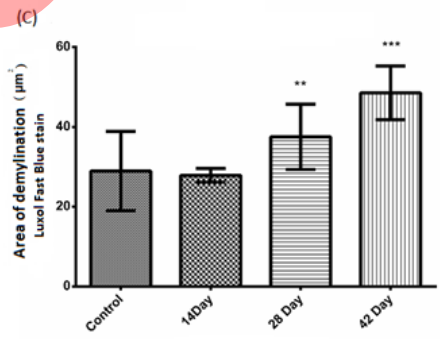


Figure 6C. After treatment, the demyelinated areas in immunized mice tissue samples were investigated and compared to the control group. The results of the analysis showed mean of 28th day was (M = 37.52, SD=8.19) and 42nd days (M=48.54, SD=6.74). Fourteen days after the immunization, and the control showed (M=29.15, SD=3.0), and (M=16.9, SD=1.98), respectively.

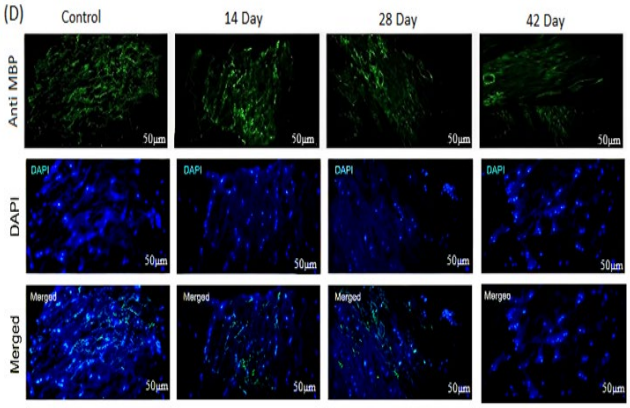


Figure 6D. anti-MBP stained spinal cord sections of rMBP-CFP immunized mice exhibited signs of reducing MBP positive area comparison to the control group.

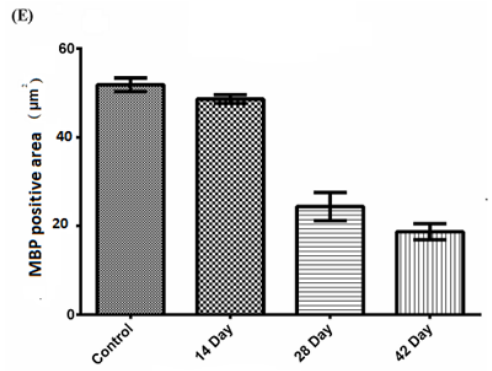
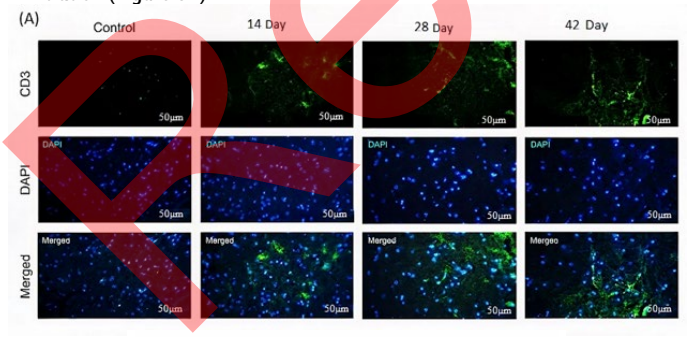


Figure 6E. The results of the analysis showed the mean of 28th day is (M = 18.67, SD=1.81) and 42nd days (M =24.35, SD=3.18). Fourteen days after the immunization, and the control showed (M=48.59, S= 0.95), and (M =51.81, SD =1.52), respectively. (**p<0.01, ***p<0. 001).

Luxol Fast Blue staining exhibited demyelinated areas in the lumbar spinal cord of rMBP-CFP immunized mice (Figure 6B). The results of Shapiro-Wilk's Test showed that the data are normally distributed. The Pearson's correlation test showed the inverse correlation between the 42nd day after immunization and the control group (p=0.042). Moreover, correlation analysis revealed a statistically significant difference between the 28th day and 42nd day in the demyelinated areas (p=0.057). It seems demyelination was accompanied by an increased level of antibodies (Figure 6C). Immunostaining anti-MBP is a marker for myelin damage, showing a decrease in the intensity of the MBP in the immunized mice compared to controls (Figure 6D). The results of Shapiro-Wilk's Test showed that the data are normally distributed. The Pearson's correlation test showed the inverse correlation between immune mice and the control group, but the p-value was not statistically significant (p=0.667) (Figure 6E).

Cell Infiltration into the CNS

To study the lymphocyte cells' infiltration into the CNS and immune cell characterization used fluorescent staining. As CD3 was present in all the stages of T cell development, it is a pan T cell marker [33]. Furthermore, CD45R is an isoform of the Ptprc gene expressed by murine B cells at all the developmental stages from pro-B cells through mature B cells [34]. The tissue infiltration of the T cell (CD3+) (Figure 7), and B cell (CD45R+) were observed primarily on inflammation areas (Figure 8A). Shapiro-Wilk's Test showed that the data are normally distributed in the T cell (CD3+). Pearson's correlation test exhibited the inverse correlation between 42 days after immunization and the control group (p=0.024) in T cell (CD3+) infiltration (Figure 7B).Shapiro-Wilk's Test showed the data are normally distributed in the B cell (CD-45R+). Pearson's correlation test revealed the inverse correlation between 42 days after immunization and the control group (p=0.046) in B cell (CD45R+) infiltration (Figure 8B).



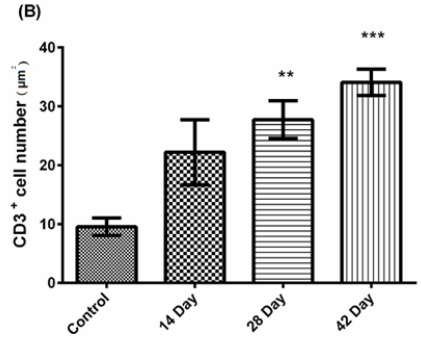


Figure 7. T cells infiltrate the lumbar spinal cord **(A)** anti-CD3 staining of the lumbar spine in control and immunizing mice by rMBP. **(B)** An increase was observed in the number of T cells (CD3+) in the lumbar spinal cord of the immunized mice. Illustrated mean of T cells (CD3+) were in the 42nd day after the treatment (M=34.7, SD = 2.24), the 28th day (M=26.90, SD=2.08), the 14th day (M=20.32, SD=3.0) and the control (M=9.55, SD=1.48). (**p < 0.01, ***p <0. 001).

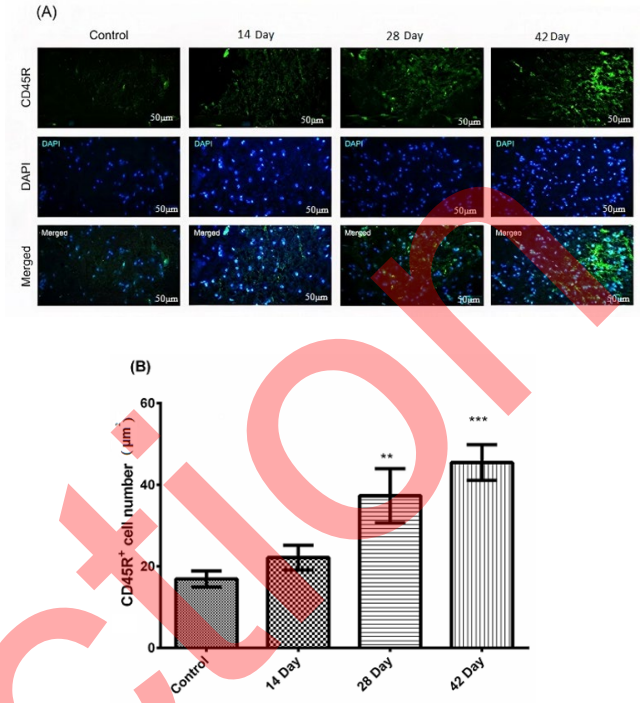
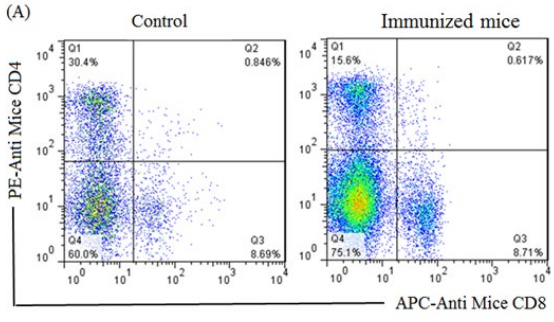


Figure 8. B cells infiltrate the lumbar spinal cord. (A) anti-CD45R staining of the lumbar spine in control and immunizing mice by rMBP.(B) An increase was observed in the number of B cells (CD45R+) in the lumbar spinal cord of the immunized mice. Illustrated mean of B cells (CD45R+) were in the 42nd day after the treatment (M=45.0, SD=4.32), the 28th day (M=37.50, SD =4.20), the 14th day (M=22.20, SD = 1.96) and the control (M=9.55, SD= 1.48). (**p <0.01, ***p <0. 001).

Cell-mediated immune response to rMBP-CFP in immunized mice

Due to the limitation of CSF in flow cytometry analysis, including low cellularity and rapid declining of WBC viability, we performed flow cytometry on splenocytes of mice. Evaluation of T CD8+ cells levels in the splenocytes showed no significant differences concerning T CD8+ cells between the immunized mice and the control (p=0.827). Evaluation of T CD4+ cells in the spleen of rMBP-CFP immunized mice indicated a statistical difference compared to the control group (p=0.050) st (Figure 9A).

The presence of B cells in the spleens of rMBP-CFP immunized mice was assessed. The splenocytes from C57BL/6J mice were stained using the CD19 and CD45R (also known as B220) antibodies to identify active B cells from conventional B cells (CD19+); both B cells expressed high levels of the CD19 molecule, but active B cells expressed higher levels of CD45R. The CD19+/CD45R+ cells in immunized mice indicated a statistical difference in the control group (p=0.050) (Figure 9B).



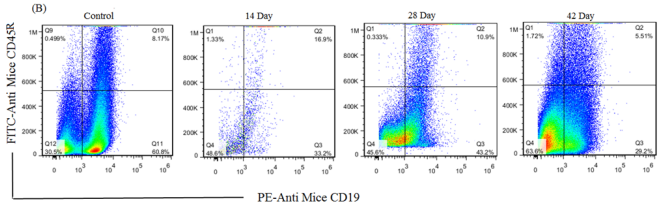


Figure 9. Cell-mediated response in rMBP-CFP immunized mice. (A) The analysis of T cells (CD4+) between control and immunized mice indicated a statistically different (p=0.050), and T cells (CD8+) are not statistically different (p=0.827). (B) The analysis of B cells (CD19+/CD45R+) cells between control and immunized mice indicated a statistically different (p= 0.050).

Correlation analysis molecular data

Pearson's correlation test showed a direct correlation between B cells (CD45R+) and T cells (CD3+) infiltration in spinal cord (p=0.009) as well as T cell (CD3+) and LFB stain (p=0.001). Moreover, the result of Pearson's correlation analysis exhibited that anti-MBP staining and B cells (CD45R+) were directly correlated (p=0.00). This data confirms a direct correlation between B cells and myelin damage. The results also revealed a significant relationship between increased immune cell infiltration into nerve tissue and myelin damage.

Pearson's correlation test between ELISA and B cells (CD45R+) on the 42nd day after treatment showed the direct correlation between increased MBP antibody levels and B cell infiltration in the spinal cord (p=0.062).

Discussion

In this research, we showed that the administration of rMBP protein could induce clinical symptoms of autoimmunity in C57BL/6J mice as a model of MS. Comparisons of antibody level and CNS histology data confirm the positive correlation between anti-myelin autoantibody and lymphocyte infiltration rate and the number of demyelinated plaques. Also, MBP autoantibody could promote inflammation and demyelination in the CNS of the mice. These findings support the previous observation of a direct interaction of autoantibody and B cells in areas of active demyelination. B cells, plasma cells, and antibodies are commonly found in active nerves lesions and have a role in the formation, maintenance, and development of new lymphoid foci in the CNS[35-37]. One of the characteristic features of MS, increased intrathecal synthesis of OCB. Early biochemical studies on autopsy brain plaques with active lesions have shown the presence of many amounts of antibodies in tissue-bound [38-40]. One of the characteristic features of MS is increased intrathecal Synthesis of OCB. Early biochemical studies on autopsy brain plaques with active lesions have shown the presence of many amounts of antibodies in tissue-bound [41].

MBP is a structural hydrophilic protein that has a critical role in the structure of myelin sheaths of oligodendrocytes Schwann cells and is found in the inner myelin layer [42]. The research demonstrated the association of the Single Nucleotide Polymorphism (SNP) rs12959006, within the MBP gene, with a higher risk of relapse MS and worse prognosis [43]. The MBP fragments are released into the CSF during demyelination, and it can be used as an index of active demyelination [44]. In active MS patients, the level of CSF MBP is frequently increased (45%-100%) and remains increased until 5 weeks to 6 weeks after the onset of symptoms [45]. Also,compared with healthy controls, increased reactivities of MBP-specific CD8+ T cells were present in MS patients[46]. These evidence indicates that MBP may be an autoantigen candidate in MS [47-50]. In conclusion, increased anti-MBP antibody levels concomitant with the penetration of inflammatory cells into nerve tissue firmly supported the role of antibodies during inflammation and nerve tissue damage. Our finding indicated a direct correlation between anti-MBP antibodies and increased infiltration of B cells into the CNS and a direct correlation with the demyelination area.

It is accepted that T cells are responsible for the initiation of MS. However, the emerging evidence about B cells and their pathological roles in autoimmune diseases has led to change in the MS treatment system [11, 51]. The success of B cell depletion therapy in MS establishes that B cells contribute to the relapsing disease stage [52]. The Rituximab, Ocrelizumab, and Ofatumumab are three therapeutic monoclonal antibodies targeting CD20+ B cells and provide consistent evidence for a disease-ameliorating effect of B cell depleting therapies in MS [53]. Targeting the antigen-specific immune response and B cell depletion are promising strategies [54]. However, the appearance of progressive multifocal upper respiratory infections and neoplasms in monoclonal-treated autoimmune diseases confirms that extended efficacy comes with clinical risks [55]. Further research to understand the immunopathological mechanism of B-cell and antibody are necessary for the treatment of MS.

Our study showed the direct correlation between the increase of B cells in the spinal cord and myelin damage, which can confirm the high importance role of B cells in damage to nerve cells. It can be concluded that targeting the humoral immune system and B cell depletion would be a reasonable therapeutic option for MS. Histopathological comparison and the humoral immune response showed increased immune cell infiltration and development of inflammatory demyelinating lesions in CNS. Reducing the anti-MBP autoantibody levels through antibody removal from patient sera and targeting B cells might be effective and promising tools for minimizing nerve tissue damages and improving the disease symptoms. In addition to the diagnostic application of these antibodies in MS, the antibody can also utilize them for designing therapeutic strategies. This strategy may prevent the progression of myelin damage in RRMS.

Conclusions

Recent advances in knowledge about B cell function and secretion of antibodies against nerve cell membranes are helpful to find efficient treatment strategies for targeting the B cell, although remaining challenges.

Ethics approval and consent to participate

The ethical approval for the study was obtained from the Iran University of Medical Sciences. This study (Number: IR.IUMS.REC 1395.9323513001) experiment and handling of mice, in general, was conducted in strict accordance with the principles outlined in the "Guide for the Care and Use of Laboratory Animals." Also, the study confirms that all experiments and handling of mice are reported under the "ARRIVE guideline."

Availability of data and materials

The data sets used and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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Authors' contributions

Maryam Sahlolbei wrote the main manuscript text and performed experiments. Maryam Hajizadeh and Marzie Naseri provided the essential mouse strains. Jafar Kiani and Zahra Madjd designed the study. Leili Saeednejad Zanjani performed the statistical analysis. All authors read and approved the final manuscript.

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Reference

1. Lee, Byung Ha, et al. "Animal models in autoimmune diseases: lessons learned from mouse models for Sjögren's syndrome." *Clin. rev. allergy immunol.* 42 (2012): 35-44.

2. Pandit, Lekha, and J. M. K. Murthy. "Treatment of multiple sclerosis." *Ann. Indian Acad. Neurol.* 14.Suppl1 (2011): S65.
3. Cepok, Sabine, et al. "Short-lived plasma blasts are the main B cell effector subset during the course of multiple sclerosis." *Brain* 128.7 (2005): 1667-1676.
4. Lublin, Fred D., and Stephen C. Reingold. "Defining the clinical course of multiple sclerosis: results of an international survey." *Neurology* 46.4 (1996): 907-911.
5. Ontaneda, Daniel, Megan Hyland, and Jeffrey A. Cohen. "Multiple sclerosis: new insights in pathogenesis and novel therapeutics." *Annu. rev. med.* 63 (2012): 389-404.
6. Bitsch, Andreas, and Wolfgang Brück. "Differentiation of multiple sclerosis subtypes: implications for treatment." *CNS drugs* 16 (2002): 405-418.
7. Wolswijk, Guus. "Chronic stage multiple sclerosis lesions contain a relatively quiescent population of oligodendrocyte precursor cells." *J. Neurosci.* 18.2 (1998): 601-609.
8. Lucchinetti, C. F., et al. "Clinical and radiographic spectrum of pathologically confirmed tumefactive multiple sclerosis." *Brain* 131.7 (2008): 1759-1775.
9. Miller, David, et al. "Clinically isolated syndromes suggestive of multiple sclerosis, part I: natural history, pathogenesis, diagnosis, and prognosis." *Lancet Neurol.* 4.5 (2005): 281-288.
10. Peterson, John W., and Bruce D. Trapp. "Neuropathobiology of multiple sclerosis." *Neurol. clin.* 23.1 (2005): 107-129.
11. Van Langelaar, Jamie, et al. "B and T cells driving multiple sclerosis: identity, mechanisms and potential triggers." *Front. immunol.* 11 (2020): 760.
12. Owens, Gregory P., et al. "The B cell response in multiple sclerosis." *Neurol. res.* 28.3 (2006): 236-244.
13. Kuenz, Bettina, et al. "Cerebrospinal fluid B cells correlate with early brain inflammation in multiple sclerosis." *PloS one* 3.7 (2008): e2559.
14. Browning, Jeffrey L. "B cells move to centre stage: novel opportunities for autoimmune disease treatment." *Nat. rev. drug discov.* 5.7 (2006): 564-576.
15. Chunder, Rittika, Verena Schropp, and Stefanie Kuerten. "B cells in multiple sclerosis and virus-induced neuroinflammation." *Front. neurol.* 11 (2020): 591894.
16. Lehmann-Horn, Klaus, Helena C. Kronsbein, and Martin S. Weber. "Targeting B cells in the treatment of multiple sclerosis: recent advances and remaining challenges." *Ther. adv. neurol. disord.* 6.3 (2013): 161-173.
17. Baecher-Allan, Clare, Belinda J. Kaskow, and Howard L. Weiner. "Multiple sclerosis: mechanisms and immunotherapy." *Neuron* 97.4 (2018): 742-768.
18. Magliozzi, Roberta, et al. "Meningeal B-cell follicles in secondary progressive multiple sclerosis associate with early onset of disease and severe cortical pathology." *Brain* 130.4 (2007): 1089-1104.
19. Kabat, Elvin A., Dan H. Moore, and Harold Landow. "An electrophoretic study of the protein components in cerebrospinal fluid and their relationship to the serum proteins." *J. clin. investig.* 21.5 (1942): 571-577.
20. Goverman, Joan. "Autoimmune T cell responses in the central nervous system." *Nat. Rev. Immunol.* 9.6 (2009): 393-407.
21. Grabar, Pierre. ""Self" and "not-self" in immunology." *Lancet* 303.7870 (1974): 1320-1322.
22. Brändle, Simone M., et al. "Distinct oligoclonal band antibodies in multiple sclerosis recognize ubiquitous self-proteins." *Proc. Natl. Acad. Sci.* 113.28 (2016): 7864-7869.
23. Smirnova, Evgeniya V., et al. "Comprehensive atlas of the myelin basic protein interaction landscape." *Biomolecules* 11.11 (2021): 1628.
24. Martinsen, Vebjørn, and Petri Kursula. "Multiple sclerosis and myelin basic protein: Insights into protein disorder and disease." *Amino Acids* 54.1 (2022): 99-109.
25. Vassall, Kenrick A., Vladimir V. Bamm, and George Harauz. "MyelStones: the executive roles of myelin basic protein in myelin assembly and destabilization in multiple sclerosis." *Biochem. J.* 472.1 (2015): 17-32.
26. Barthelmes, Julia, et al. "Induction of experimental autoimmune encephalomyelitis in mice and evaluation of the disease-dependent distribution of immune cells in various tissues." *JoVE (J. Vis. Exp.)* 111 (2016): e53933.
27. Amor, Sandra, and David Baker. "Checklist for reporting and reviewing studies of experimental animal models of multiple sclerosis and related disorders." *Mult. Scler. Relat. Disord.* 1.3 (2012): 111-115.
28. Faunce, Douglas E., Ania Terajewicz, and Joan Stein-Streilein. "Cutting edge: in vitro-generated tolerogenic APC induce CD8+ T regulatory cells that can suppress ongoing experimental autoimmune encephalomyelitis." *J. Immunol.* 172.4 (2004): 1991-1995.
29. Tsai, C. Y., et al. "Intracerebral injection of myelin basic protein (MBP) induces inflammation in brain and causes paraplegia in MBP-sensitized B6 mice." *Clin. Exp. Immunol.* 109.1 (1997): 127-133.
30. Shaw, Michael K., et al. "A combination of adoptive transfer and antigenic challenge induces consistent murine experimental autoimmune encephalomyelitis in C57BL/6 mice and other reputed resistant strains." *J. neuroimmunol.* 39.1-2 (1992): 139-149.
31. Feldman, Ada T., and Delia Wolfe. "Tissue processing and hematoxylin and eosin staining." *Histopathol.: Methods Protoc.* (2014): 31-43.
32. Stromnes, Ingunn M., and Joan M. Goverman. "Active induction of experimental allergic encephalomyelitis." *Nat. protoc.* 1.4 (2006): 1810-1819..
33. Koduri, Megha Pallavi. The curative potential of chimeric antigen receptor T-cell therapy for B-cell malignancies. *Diss, Boston Univ.*, 2017. (2022): 105696.
34. Machado-Santos, Joana, et al. "The compartmentalized inflammatory response in the multiple sclerosis brain is composed of tissue-resident CD8+ T lymphocytes and B cells." *Brain* 141.7 (2018): 2066-2082.
35. Lyons, Jeri-Anne, et al. "Critical role of antigen-specific antibody in experimental autoimmune encephalomyelitis induced by recombinant myelin oligodendrocyte glycoprotein." *Eur. j. immunol.* 32.7 (2002): 1905-1913.

36. Genain, Claude P., et al. "Identification of autoantibodies associated with myelin damage in multiple sclerosis." *Nat. med.* 5.2 (1999): 170-175.
37. Moore, G. R., and C. S. Raine. "Immunogold localization and analysis of IgG during immune-mediated demyelination." *Lab. Investig.; J. Tech. Methods Pathol.* 59.5 (1988): 641-648.
38. Weber, Bernhard Hemmer, and Sabine Cepok. "The role of antibodies in multiple sclerosis." *Biochim. Biophys. Acta (BBA)-Mol. Basis Dis.* 1812.2 (2011): 239-245.
39. McMillan, R., R. Longmire, and R. Yelenosky. "The effect of corticosteroids on human IgG synthesis." *J. Immunol.* 116.6 (1976): 1592-1595.
40. Stork, L., et al., Differences in the reponses to apheresis therapy of patients with 3 histopathologically classified immunopathological patterns of multiple sclerosis. *JAMA neurol.*, 2018. 75(4): p. 428-435.
41. Mehta, P. D., et al. "Bound antibody in multiple sclerosis brains." *J. Neurol. Sci.* 49.1 (1981): 91-98.
42. Glushakova, Olena Y., et al. "The use of blood-based biomarkers to improve the design of clinical trials of traumatic brain injury." *Handb. neuroemergency clin. trials, Acad. Press*, 2018. 139-166..
43. Espino-Paisán, Laura, et al. "A polymorphism within the MBP gene is associated with a higher relapse number in male patients of multiple sclerosis." *Front. immunol.* 11 (2020): 771.
44. Giovannoni, Gavin. "Cerebrospinal fluid analysis." *Handb. clin. neurol.* 122 (2014): 681-702.
45. Lamers, , H. PM de Reus, and P. JH Jongen. "Myelin basic protein in CSF as indicator of disease activity in multiple sclerosis." *Mult. Scler. J.* 4.3 (1998): 124-126.
46. Ji, Qingyong, Antoine Perchellet, and Joan M. Goverman. "Viral infection triggers central nervous system autoimmunity via activation of CD8+ T cells expressing dual TCRs." *Nat. immunol.* 11.7 (2010): 628-634.
47. Riedhammer, Christine, and Robert Weissert. "Antigen presentation, autoantigens, and immune regulation in multiple sclerosis and other autoimmune diseases." *Front. immunol.* 6 (2015): 322.
48. Ota, Kohei, et al. "T-cell recognition of an immuno-dominant myelin basic protein epitope in multiple sclerosis." *Nature* 346.6280 (1990): 183-187.
49. Chou, Y. K., et al. "Response of human T lymphocyte lines to myelin basic protein: association of dominant epitopes with HLA class II restriction molecules." *J. neurosci. res.* 23.2 (1989): 207-216.
50. Richert, John R., et al. "Expansion of antigen-specific T cells from cerebrospinal fluid of patients with multiple sclerosis." J. neuroimmunol. 5.3 (1983): 317-324.
51. Hampe, Christiane S. "B cells in autoimmune diseases." *Scientifica* 2012 (2012).
52. Klein da Costa, B., et al. "Unraveling B lymphocytes in CNS inflammatory diseases: Distinct mechanisms and treatment targets." *Neurology* 95.16 (2020): 733-744.
53. Gasperi, Olaf Stüve, and Bernhard Hemmer. "B cell-directed therapies in multiple sclerosis." *Neurodegener. dis. manag.* 6.1 (2016): 37-47.
54. Musette, Philippe, and Jean David Bouaziz. "B cell modulation strategies in autoimmune diseases: new concepts." Front. immunol. 9 (2018): 622.
55. Florou, Despoina, et al. "Anti-CD20 agents for multiple sclerosis: spotlight on ocrelizumab and ofatumumab." *Brain Sci.* 10.10 (2020): 758.

Retraction Note

The Publisher and Editor regretfully retract the article titled "Role of B Cells and Myelin Basic Protein-Induced Antibodies in the Pathogenesis of Experimental Autoimmune Encephalomyelitis in C57bl/6 J Mice " published in Journal "Journal of Multiple Sclerosis" Volume 10, Issue 4, and Page no. 1-8. Following an investigation which found that the author violated the Journal's policy and putting false allegations towards to the journal. This is contrary to the ethical standards of the journal and unacceptable. The author denied to support open access. The authors have been notified of this decision. The Publisher and Editor apologize to the readers of the journal for any inconvenience this may cause.

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