Intraperitoneal injection of mesenchymal stem cells-conditioned media (MSCS-CM) treated monocyte can potentially alleviate motor defects in experimental autoimmune encephalomyelitis female mice; an original experimental study

Bahman Jalali Kondori  
Baqiyatallah University of Medical Sciences

Amir Abdolmaleki  
Baqiyatallah University of Medical Sciences

Mahdi Raei  
Baqiyatallah University of Medical Sciences

Hadi Esmaeili Gouvarchin Ghaleh  
h.smaili1401@gmail.com  
Baqiyatallah University of Medical Sciences

Research Article

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Abstract

**Introduction:** Multiple sclerosis (MS) is destructive pathology of myelin in central nervous system (CNS) leading to occurrence of physical complications. Experimental autoimmune encephalomyelitis (EAE) is the laboratory model of MS widely used for CNS-associated inflammatory research. Cell therapy using macrophage M2 (MPM2), as a cell type with anti-inflammatory feature, seems useful for EAE treatment. This study was aimed to investigate the therapeutic anti-inflammatory effects of intraperitoneal (IP) injection of MPM2 cells on alleviation of motor defect in female EAE-affected mice.

**Materials and Methods:** 24 C57/BL6 female mice were divided into EAE (EAE-affected mice), EAE+Dexa (EAE-affected mice receiving dexamethasone), EAE+PBS (EAE-affected mice receiving PBS) and EAE+MP2 (EAE-affected mice receiving MPM2). EAE was induced through application of spinal homogenate of guinea pigs. MPM2 cells were extracted and cultured from bone marrow and injected (10^6 cells/ml) in three days of 10, 13 and 16 post-immunization (p.i). The clinical score (CS), anti-inflammatory cytokines, pro-inflammatory gene expression and histopathological investigations were considered. Data were assessed analyzed using SPSS software (v.19) and p<0.05 was assumed significant level.

**Results:** IP injection of MPM2 in EAE-affected mice can significantly (p<0.05) alleviate CS index, accelerate anti-inflammatory cytokines (IL-4, IL-10), and attenuate pro-inflammatory genes expression (TNF-α, IL-1β). These findings were also approved by histopathological tissue staining.

**Conclusion:** IP injection of MPM2 to the EAE-affected mice can potentially reduce the CNS inflammation, neuronal death and myelin destruction leading to the improvement of animal movement behaviors.

**Introduction**

MS is a chronic autoimmune pathology with the basis of vast neural inflammation affects CNS and damage to myelin sheath (1). The etiology of MS is not fully understood, but it is believed to be caused by a combination of genetic and environmental factors. Epidemiological studies have shown that MS is more common in women than men and typically affects individuals between the ages of 20 and 40 (2). EAE is one of main animal model of multiple sclerosis (MS) which is commonly used for investigation of pathophysiology and the treatment of MS (3). EAE is induced by injection of myelin antigens (such as myelin basic protein or proteolipid protein) along with an adjuvant to stimulate the immune system function. EAE can be induced in various animal species, including mice, rats, guinea pigs, rabbits, and monkeys (4). The symptoms of this experimental model completely depends on the animal species and the type of myelin antigen used for pathology induction. Common symptoms include paralysis, ataxia, tremors, weakness, and sensory deficits. EAE is characterized by inflammation and demyelination in CNS, which leads to axonal damage and neurological dysfunction (5). The immune system attacks myelin antigens in the CNS, causing an inflammatory response that damages the myelin sheath around nerve fibers. EAE follows a relapsing-remitting course similar to MS in humans (6). Overall, the EAE is a
valuable animal model for studying the pathophysiology and treatment of MS. Cell therapy has emerged as a promising approach for the treatment of MS in animal models or clinical trials. In experiments, the cell therapy has been shown to promote the regeneration of damaged nerve cells and reduce CNS inflammation (7). The application of stem cells (SCs), particularly the mesenchymal SCs, has shown promising results in both animal models and clinical trials (8). These cells contain the ability to differentiate into various cell types and release anti-inflammatory factors. While more research is needed to fully understand the mechanisms underlying cell therapy for MS treatment, it holds great potential as a novel therapeutic approach for this debilitating disease. MPM2 is a type of immune cell which possesses unique cellular features and anti-inflammatory characteristics (9). These cells are known to play a crucial role in regulation of the immune response and promotion of tissue repair. MPM2s are characterized by their ability to produce anti-inflammatory cytokines, such as IL-10 and TGF-β, which help to suppress inflammation and promote tissue healing (10). These cells can directly suppress the activity of pro-inflammatory immune cells, such as Th1 and Th17 cells (11) and promote the proliferation and differentiation of regulatory T cells, which help to maintain immune tolerance and prevent autoimmunity (12). Finally, these cells can secrete growth factors and cytokines that promote tissue repair and regeneration (13). In this experimental animal study, the authors aimed to evaluate the role of anti-inflammatory features of IP injection of MPM2 into the female mice with EAE. The therapeutic levels of cell therapy using MPM2 were assessed using genetic (through the assessment of pro-inflammatory genes expression of TNF-α and IL-1β), serological (through the assessment of anti-inflammatory cytokines of IL-4 and IL-10), and histopathological (through the tissue staining by hematoxylin and eosin, Luxol fast blue, and Nissl), and serological investigations.

Materials and methods

Ethical consideration

All experimental protocols performed on animals were based on the Helsinki guidelines and were carried out under the supervision of the BMSU (Ethic NO: 400000308). All animal manipulation was applied gently and the animals were anesthetized in all invasive interventions. Normal living conditions were totally prepared including temperature, humidity, light, and access to water and food (14).

Chemicals and agents

All chemicals for laboratory assessment were provided from Sigma-Aldrich Co. (Merck SA, an affiliate of Merck KGaA, Darmstadt, Germany) and other Iranian Laboratory Chemical Company with the associated CAT number.

Animals and study grouping

Adult (8–12 weeks) female C57/BL6 mice (~ 30gr) were divided into 4 different groups (n = 6 in each); EAE animals (or disease control) included the EAE animals received no experimental treatment, EAE + Dexa group (or treatment control) included the EAE animals received dexamethasone (Dexa), EAE + PBS
group (or vehicle solvent) included the EAE animals received PBS as the solvent, and EAE + MPM2 group (or main treatment) included the EAE animals received IP injection of MPM2 with the total concentration of \(10^6\) cell/ml in three different routes (days of 10, 13, and 16).

**Induction of EAE**

The active EAE was induced using subcutaneous immunization through injection of pig spinal cord emulsion containing myelin glycoproteins and Freund's complete adjuvant (CFA). In this process, the guinea pig received light sedation using intramuscular injection of Ketamine/xylazine. Then, the animals were euthanized by inhalation of chloroform. The animals were dissected and the vertebral column was isolated. The spinal cord was excisioned and treated by liquid nitrogen (10 min). Then, the samples were pounded with a mortar followed by vortex for tissue homogenate preparation. 50ml of incomplete Freund's adjuvant (IFA) was mixed with 200mg of dried Mycobacterium Tuberculosis H37Ra (MT) with a concentration of 4mg/ml to obtain CFA. Then, with a ratio of 1tissue homogenate/1CFA, it was poured into the Falcon and treated under Vortex until the homogeneous tissue with the pink color was obtained. Finally, 0.4ml of the prepared suspension was injected subcutaneously in the neck area of C57/BL6 mice. Pertussis toxin was diluted at 1µg/µlPBS on days of 0 and 2 p.i (200ng for each injection). Usually the clinical score representing movement defects were appeared between days of 10 and 28 p.i (15).

**Animal weighing and CS index assessment**

In the whole research process, the animals were weighed prior to induction until the last day of experiment. To rate the level of neural damage, the CS index as a criterion representing motor defect of limbs was hired including; 0 = no symptoms, 1 = tail paralysis, 2 = complete paralysis of one dorsal limb or partial paralysis of two dorsal limbs, 3 = complete paralysis of both dorsal limbs. 4 = complete paralysis of both back and front limbs, and 5 = death (16).

**MPM2 culture and cell therapy application procedure**

Immunized C57BL/6 mice were euthanized and bone marrow cells were collected (from femoral and tibial bones) in RPMI-1640 complete medium (GIBCO, Waltham, MA, USA) on days of 2, 5 and 8 p.i. Red blood cell were lysed and remaining were cultured in RPMI-1640 complete medium supplemented with penicillin and streptomycin (1%; Hyclone, Logan, UT, USA), fetal bovine serum (10%; Sigma Aldrich), interleukin (IL)-4 (10 ng/ml; PeproTech, Rocky Hill, NJ, USA) and macrophage colony-stimulating factor (M-CSF) (10 ng/ml; PeproTech). On days 3, 5 and 7 post-culture, half the previous medium was replaced with fresh medium and the concentrations of IL-4 and M-CSF were increased to 20 ng/ml. M2 macrophages were harvested on day 8 post-culture using ethylenediamine tetraacetic acid (EDTA; Sigma). Then, the cell concentration was increased to \(10\times10^6\)/ml and injected IP on days of 10, 13 and 16 p.i. Animals were given 28 days for complete implantation of the injected cells. The process of MPM2 preparation was conducted by the Tehran Cell Culture Company (17).

**Tissue dissection and sampling**
On the 28th day of treatment the tissue dissection and sampling was conducted. The animals were anesthetized by intramuscular injection of ketamine/xylazine for induction of light sedation and then they were euthanized by cervical dislocation. Blood samples were aspirated through cardiac puncture and centrifuged (5000 rpm, 15 min) for blood serum collection. Then, a midsagittal skin incision was made on the cranium of animals and the brain was removed by Total Brain Dissection method. Brain tissue samples were fixed in 10% formalin for histopathological staining and were conserved in liquid nitrogen for future genetic evaluations (18).

**Histopathological staining of H&E, LFB, and Nissl**

Following 10 days of brain tissue preservation in formalin 10%, the samples were processed by the tissue processor. For all staining types, the process of deparaffinization was applied by 3 containers of xylol (5min for each). Tissue dehydration was also conducted using ethyl alcohol (100–70%, 5min for each stage). After each specific staining, the slides were treated for dehydration using ethyl alcohol (3min for each) with increasing concentration (70, 80, 90% and absolute alcohol). To clarify the samples, the slides were placed in three containers containing Xylol (each for 3 minutes). For H&E staining, the slides were placed in hematoxylin (15 min) and followed by washing in running water (5min). Also, to stabilize the color of the nuclei, the slides were placed in lithium carbonate (3min). Also, the eosin was used for slide staining (5-10min) and then were washed in running water (2-3min) (19). LFB was used for myelin sheath lipoproteins staining in blue. Routine deparaffinization, and hydration were also applied. Tissue sections were located in LFB staining overnight and then were then differentiated using lithium carbonate solution (30s). Excess color was also rinsed by 95% alcohol solution followed by running water (2min) (20). Nissl staining was hired to visualize masses of rough endoplasmic reticulum and free polyribosomes, as well as nuclear DNA staining found in nerve cells distinguishing the neural cells from glia. Thus, the architectural structure of neural cells can be examined well. The loss of Nissl substance indicated abnormalities such as damage or cell analysis following EAE induction. For this purpose, following routine slides processing, the slides were washed in running water (2min) and placed in 0.1% Cresyl (30min) (21).

**Pro-inflammatory genes expression assessment of TNF-α and IL-1β**

This method was used to evaluate the pro-inflammatory gene expression including TNF-α (22) and IL-1β (23). The related sequences of primers used for gene detection are listed in Table 1. All brain tissues were stored in liquid nitrogen to prevent tissue degeneration. Total RNA contents were extracted using RNeasy mini kit (Qiagen Co.). cDNA was produced from the RNA extraction utilizing RevertAid™ First Strand cDNA Synthesis Kit (Fermentas). Also, the DNA samples were treated by DNase set kit (Qiagen). The expression levels of pro-inflammatory genes were measured through glyceraldehyde-3-phosphate dehydrogenase primer as endogenous control by SYBR Green through the comparative method. ACTB was also considered as internal control.
Table 1
Sequence of forward and reverse primers, annealing temperature and length of the fragment.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequences (5’–3’)</th>
<th>Length (bp)</th>
<th>Tm</th>
<th>Annealing temp</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>F CTTCAGGGATATGTGATGGACTC</td>
<td>20</td>
<td>58.53</td>
<td>58</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>R GGAGACCTCTGGGGGAGATGT</td>
<td>19</td>
<td>58.32</td>
<td></td>
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<tr>
<td>IL-1β</td>
<td>F CCACAGACCTTCCAGGAGAATG</td>
<td>18</td>
<td>59.43</td>
<td>52</td>
<td>150</td>
</tr>
<tr>
<td></td>
<td>R GTGCAGTTCAGTGATCGTACAGG</td>
<td>18</td>
<td>59.32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACTB</td>
<td>F TGACCAGATCATGTGGAGACC</td>
<td>23</td>
<td>60.31</td>
<td>60</td>
<td>146</td>
</tr>
<tr>
<td></td>
<td>R CTCGTAGATGGGCCACAGTGCGG</td>
<td>23</td>
<td>64.94</td>
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</tbody>
</table>

Serological assessment of anti-inflammatory cytokines of IL-4 and IL-10

IL-4 (24) and IL-10 (25) serum concentrations were measured by ELISA technique (CK-E30634 kit, Biopharm, USA). Following preparation of the samples and associated standards, 40µl of the serum sample was added to each well of microplate coated with monoclonal antibodies, followed by 10µL of biotin solution and 50µl of HRP linked polyclonal antibody. The microplate was then placed at 37°C for 60min. Then they were washed with the washing solution (5times), and the chromogen A and B solutions were added to the wells and incubated at 37°C for 10min. 50µl of Stop solution was added to the wells for color resuscitation. Finally, the optimum density was determined at the 450 nm wavelength using a Microplate Reader and the concentration of the cytokines was expressed in ng/L.

Statistical analysis

The normal distribution of the extracted data was evaluated by Kolmogorov-Smirnov statistical analysis. Unpaired t-test and ANOVA using SPSS software (v.16) were used to investigate significant changes between two and several groups, respectively. The data were reported as Mean ± SD and p-value < 0.05 was considered as a significant level of difference between groups.

Results

Weight-associated alteration

In all groups, no significant changes (p > 0.05) was found from the day 0 to 16 regarding the weight of animals. From the 17th to the 28th days of treatment, significant (p < 0.05) changes of the weight of animals were detected as follows; the weight was increased significantly (p < 0.05) in EAE + Dexa and EAE + MPM2 groups compared to EAE and EAE + PBS animals, meanwhile, there were no significant weight changes was found among the EAE and EAE + PBS groups, as well as between the EAE + Dexa and EAE + MP2 groups (p > 0.05) (Fig. 1).
CS-associated alteration

CS index represented the range of motor defect in EAE animals; the criterion of CS = 0 indicated healthy status and CS = 5 represented the animal death caused by EAE. All animals had no signs of obvious motor defect by the days of 10 or 11 p.i. The obvious motor defect appeared from the 11th to 16th or 17th days of experiment. Significant incremental differences (p < 0.05) were seen in CS index from the day 17 to 28 between the EAE and EAE + PBS groups with EAE + Dexe and EAE + MP2 animals and significant (p < 0.05) decremented trend was also detected in the EAE + Dexe and EAE + MP2 groups than EAE and EAE + PBS animals. In days 25–28, the CS was significantly (p < 0.05) decreased in the EAE + MP2 group compared to the EAE + Dexe animals. No death following the induction of EAE (Clinical score = 5) was detected in the experiment (Fig. 2).

Histopathological changes following H&E staining

H&E staining of brain samples in the midsagittal section of the corpus callosum showed many obvious histopathological changes. The density of cell nuclei in this area was found microscopically lower in in EAE and EAE + PBS animals than EAE + Dexe and EAE + MP2 groups. Also, H&E staining in this area indicated the density of white matter fibers. Examining the brain cortex with H&E staining sometimes showed a higher density of cell death in the EAE and EAE + PBS groups than in the EAE + MP2 and EAE + Dexe groups (Fig. 3).

Histopathological changes following LFB staining

LFB staining specifically appear tissue changes in the white matter of CNS. Microscopic studies in midsagittal sections of corpus callosum showed a noticeable decrease in white matter density in the EAE and EAE + PBS groups compared to the EAE + Dexe and EAE + MP2 animals. This reduction in the density of nerve fibers can probably be a product of neuronal damage following the induction of EAE (Fig. 4).

Histopathological changes following Nissl staining

Microscopic examination of midsagittal brain tissue sections following Nissl staining showed the presence of multiple cell gaps in EAE and EAE + PBS groups compared to EAE + Dexe and EAE + MP2 treatment groups. Also, the images showed an increase in the density of white matter in the corpus callosum after treatment with dexamethasone and MP2 in the EAE + Dexe and EAE + MP2 groups compared to the EAE and EAE + PBS control groups (Fig. 5).

Alteration of pro-inflammatory gene expression of TNF-α and IL-1β
TNF-α and IL-1β pro-inflammatory genes expression were significantly (p < 0.05) increased in EAE and EAE + PBS groups compared to EAE + Dexe and EAE + MP2 treatment animals. In addition, although the level of TNF-α and IL-1β genes expression in EAE + MP2 group was lower than EAE + Dexe, but this decrease was not found to be significant (Fig. 6).

**Alteration of anti-inflammatory cytokines of IL-4 and IL-10**

Evaluations of IL-4 and IL-10 serum concentrations showed that the concentration of these cytokine was found higher significantly (p < 0.05) in EAE and EAE + PBS than the animals in EAE + Dexe and EAE + MP2 treatment groups (Fig. 7).

**Discussion**

Macrophages are a type of immune cell that play a crucial role in the innate immune response. They are divided into two subtypes, M1 and M2, which have distinct functions. Recent research has also identified an M3 switch phenotype (26). In MS, increased macrophages have been associated with neuronal damage. During the acute phase of MS, M1 macrophages are activated and release pro-inflammatory cytokines, leading to neuro-inflammation, CNS demyelination, and neuronal death (27). However, as the disease progresses, M2 macrophages gradually increase and become dominant in the CNS. These cells release anti-inflammatory cytokines that reduce inflammation and promote tissue repair while inhibiting further progression of the disease. In late stages of MS, M2 macrophages play a crucial role in reducing inflammation and promoting tissue repair. The results of previous studies support these findings (28).

Recent research has shown that certain treatments for MS, such as glucocorticoids, fingolimod, glatiramer acetate, teriflunomide, and IFN-β, can have an impact on the expression of M1 macrophages or the activation of M2 macrophages. M1 macrophages are known to promote inflammation, while M2 macrophages have anti-inflammatory properties (29). Previous studies have demonstrated that therapy with M2 macrophages or microglia can prevent the onset of type 1 diabetes and the damage caused by spinal cord injuries, as well as alleviate symptoms of EAE in rats (30). However, there is a limited supply of macrophages available for use in therapy, and alternative methods are necessary to obtain an adequate number of macrophages. In recent years, researchers have utilized cytokines and compounds that can stimulate the differentiation of bone marrow stem cells into M2 macrophages. This approach has been successful in transforming stem cells into M2 macrophages (10). In a recent study, IL-4 and M-CSF were used to induce bone marrow stem cells to differentiate into M2 macrophages. These M2 macrophages were then transferred into EAE mice at an early stage of the disease. The results showed that the M2-treated group experienced a simultaneous development of symptoms but had alleviated symptoms at the peak of EAE, and their symptom relief was faster than the control group. Overall, this study suggests that inducing bone marrow stem cells to differentiate into M2 macrophages could be a promising therapeutic approach for MS and other autoimmune diseases. The study found that the group treated with M2 macrophages induced from bone marrow stem cells had lower levels of spinal cord inflammation, inflammation and demyelination in the spinal cord, and inflammatory factors secretion in the blood compared to the control group (31). This result is consistent with previous studies that have
used M2 macrophages for therapeutic purposes. However, this study differs in that it utilized M2 macrophages induced from bone marrow stem cells and administered them intravascularly, which is more practical for clinical use. Furthermore, the study found that after M2 macrophage treatment, the number of macrophages in the spleen decreased, including both M1 and M2 macrophages, but the decrease was more significant in M2 macrophages. Additionally, pro-inflammatory factors were reduced while anti-inflammatory factors were increased in peripheral blood. These findings suggest that inducing bone marrow stem cells to differentiate into M2 macrophages could be a promising therapeutic approach for MS and other autoimmune diseases (32). The use of intravascular administration and the reduction of both M1 and M2 macrophages in the spleen may also have important clinical implications for future treatments. The immune system plays a critical role in maintaining internal environment homeostasis, and it does so by activating various mechanisms to restore balance once the inflammatory reaction occurs. In our study, we injected a large number of M2 macrophages into EAE mice and observed a decline in macrophage activation which helped restore the internal environment homeostasis. This was accompanied by a significant increase in anti-inflammatory factors in the blood, which facilitated tissue repair and alleviated EAE symptoms. While no other study has used macrophages to treat multiple sclerosis, it is important to note that human and mouse macrophages differ significantly. In mice, M1 macrophages express iNOS and CD40, while M2 macrophages express CD206 and Arg-1 (33). However, in humans, M1 macrophages preferentially express iNOS, CD40, and NOX1, while M2 macrophages preferentially express CD206 and CD163. Furthermore, human macrophages can exhibit an intermediate polarization phenotype with co-expression of both M1 and M2 marker proteins (34). Recent studies have also identified regulatory macrophages (MRs) that play a key role in inflammation response by restricting innate and adaptive immune processes. In atherosclerosis, additional macrophage phenotypes have been described, including M4 macrophages induced by CXCL4, Mox macrophages induced by oxidized LDL, and M (Hb) macrophages induced by hemoglobin in cases of intraplaque hemorrhage (also known as Mhem macrophages). These findings suggest that future research should consider the diversity of macrophage phenotypes when developing therapeutic approaches for autoimmune diseases like MS (35). NF-κB is a crucial intracellular signaling molecule that regulates the expression of various molecules involved in immune and inflammatory responses. It exists mainly in the form of p50/p65 isodimers, and p65 plays a significant role in the onset and progression of multiple sclerosis (MS). Studies have shown that alterations in the activity or protein expression of NF-κB are closely related to the severity of experimental autoimmune encephalomyelitis (EAE), a widely used animal model for MS. Mice with NF-κB defects have been found to exhibit reduced disease incidence, clinical symptoms, and CNS inflammation. Additionally, NF-κB is also crucial in activating and differentiating T cells, and blocking its function has been shown to be an effective strategy for preventing autoimmune encephalitis (36). NF-κB activation can occur through various pathways, including Toll-like receptors (TLRs), cytokine receptors, and antigen receptors. In the context of EAE and MS, NF-κB activation is primarily mediated by TLRs, which recognize pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) released by damaged tissues. This leads to the production of pro-inflammatory cytokines such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6), which contribute to the development of CNS
inflammation and demyelination (37). In addition to its role in immune and inflammatory responses, NF-κB also plays a critical role in regulating cell survival and apoptosis. It has been shown to promote cell survival in various cell types, including neurons and oligodendrocytes, which are particularly vulnerable in MS. Conversely, inhibition of NF-κB activity has been found to induce apoptosis in these cells and exacerbate MS-like pathology in animal models. These findings suggest that NF-κB may be a potential therapeutic target for MS, although careful consideration must be given to its multiple roles in different cell types and physiological processes (38). The researchers observed that the symptoms of mice with EAE were reduced after the application of M2 macrophages, and this was accompanied by a reduction in NF-κB expression in the spinal cord. This led them to investigate whether NF-κB was involved in the polarization of M1 macrophages to M2 macrophages. Previous studies have shown that the NF-κB signal transduction pathway plays a role in the inflammatory immune response induced by macrophages. Specifically, NF-κB activity is closely related to the number, state, degree, and product of microglial activation. This means that higher NF-κB activity leads to more activated microglial cells and increased production of inflammatory factors (39). Based on these findings, the researchers hypothesized that blocking the NF-κB pathway could reduce the polarization of M1 macrophages, increase the number of M2 macrophages, and alleviate the symptoms of EAE (36). This hypothesis is supported by previous research showing that blocking NF-κB function can be an effective strategy for preventing autoimmune encephalitis. However, it is important to note that NF-κB also plays a critical role in regulating cell survival and apoptosis, particularly in vulnerable cell types such as neurons and oligodendrocytes in MS. Therefore, any potential therapeutic targeting of NF-κB must be carefully considered in light of its multiple roles in different cell types and physiological processes. To further investigate the role of NF-κB in the polarization of M1 and M2 macrophages, the researchers applied a NF-κB blocker called BAY-11-7082 in both in vitro and in vivo experiments (40). This blocker works by inhibiting the phosphorylation of NF-κB65, thereby blocking its activity. The results showed that the severity of EAE was alleviated after the application of the blocker, and the therapeutic effect was better in the prevention group than in the treatment group. This suggests that early blockade of NF-κB is more effective in reducing the symptoms of EAE. Further analysis of macrophages in the spleen of mice at the peak of EAE showed that the proportion of total macrophages was lower in the NF-κB blockade group than in the control group. Additionally, the proportion of M1 macrophages declined significantly, while the proportion of M2 macrophages decreased somewhat. This suggests that inhibition of the NF-κB pathway can specifically inhibit the generation of M1 macrophages, which are proinflammatory, while promoting the generation of M2 macrophages, which are anti-inflammatory (41). In vitro experiments also revealed that the blocker blocked the activity of both M1 and M2 macrophages simultaneously. However, the blockade of activity of M1 macrophages was more obvious than activity of M2 macrophages, which is consistent with the results from the in vivo study. Overall, these findings suggest that inhibition of the NF-κB pathway can effectively reduce inflammation in EAE by inhibiting the generation of M1 macrophages and proinflammatory factors. Early addition of this inhibitor could block the proinflammatory effect of M1 macrophages more effectively and reduce the inflammatory response in EAE mice. However, it is important to consider the potential effects on other cell types and physiological processes before using NF-κB inhibitors as a therapeutic strategy. The present study
provides new insights into the role of macrophages in the occurrence and development of EAE. Specifically, the study found that M1 macrophages have proinflammatory effects, while M2 macrophages have anti-inflammatory effects. This suggests that macrophage polarization plays a crucial role in the pathogenesis of EAE and other autoimmune diseases (42). Moreover, the study demonstrated that bone marrow stem cells can be successfully induced into M2 macrophages, which could have promising therapeutic effects on EAE and potentially other autoimmune diseases. The ability to induce M2 macrophages from stem cells could enable rational therapeutic approaches for these diseases in the future. The study also revealed that NF-κB blockade effectively prevented EAE development and alleviated its severity by blocking the NF-κB pathway. This finding suggests that inhibition of the NF-κB pathway could be a viable therapeutic strategy for reducing inflammation in EAE and potentially other autoimmune diseases. However, further research is needed to fully understand how the NF-κB pathway is involved in the process of macrophage polarization and to assess the potential effects on other cell types and physiological processes before using NF-κB inhibitors as a therapeutic approach (43).

Conclusion

This experimental study revealed a new modern insight into the application of MPM2 on the side-effects of EAE. The findings showed that IP application of MPM2 through a process of cell therapy can inhibit the neuro-inflammation leading to the neuro-regeneration. MPM2 application also down-regulates the expression of pro-inflammatory genes and up-regulates anti-inflammatory cytokines. These useful changes can finally lead to the decreased levels of clinical score and low levels of motor defects. Totally, more studies are strictly needed to clear the exact molecular role of MPM2 on alleviation of EAE for pre-clinical application in human MS.

Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>MP2</td>
<td>Macrophage M2</td>
</tr>
<tr>
<td>MP1</td>
<td>Macrophage M1</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
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<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalomyelitis</td>
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<td>BM</td>
<td>Bone marrow</td>
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</table>

Declarations

Author contributions
The main design of this study was proposed by HEGGH. AA and BJK were responsible for reviewing studies and data collection. All statistical analyses were performed by AA and MR. The primary manuscript was prepared by AA and approved by all authors.

**Funding**

This study was approved by Baqiyatallah University of Medical Sciences (400000308), and this university was responsible for funding provision.

**Data availability**

The datasets used and analyzed for this are available from the corresponding author upon reasonable request.

**Conflict of Interest**

The authors declare that they have no competing interests.

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Figures
Figure 1

Weight changes of the animals in treatment groups (n=6 animals in each groups) in 4 weeks of experiment. EAE; experimental autoimmune encephalomyelitis, Dexe; dexamethasone, PBS; Phosphate-buffered saline, MPM2; macrophage M2. * represented significant changes.
Figure 2

Clinical score changes of the animals in treatment groups (n=6 animals in each group) in 4 weeks of experiment. EAE; experimental autoimmune encephalomyelitis, Dexasone, PBS; phosphate-buffered saline, MPM2; macrophage M2. * represented significant changes.
Figure 3

Histopathological changes (Hematoxylin and Eosin staining) of the brain of animals in treatment groups (n=6 animals in each groups) in midsagittal section. EAE; experimental autoimmune encephalomyelitis, Dexa; dexamethasone, PBS; Phosphate-buffered saline, MPM2; macrophage M2.
Figure 4

Histopathological changes (Luxol Fast Blue staining) of the brain of animals in treatment groups (n=6 animals in each groups) in midsagittal section. EAE; experimental autoimmune encephalomyelitis, Dexe; dexamethasone, PBS; Phosphate-buffered saline, MPM2; macrophage M2.
Figure 5

Histopathological changes (Nissl staining) of the brain of animals in treatment groups (n=6 animals in each groups) in midsagittal section. EAE; experimental autoimmune encephalomyelitis, Dexa; dexamethasone, PBS; Phosphate-buffered saline, MPM2; macrophage M2.
mRNA fold change of TNF-α and IL-1β genes expression of the brain of animals in treatment groups (n=6 animals in each groups). EAE; experimental autoimmune encephalomyelitis, Dexe; dexamethasone, PBS; Phosphate-buffered saline, MPM2; macrophage M2. *** represented significant changes.

Figure 6
Figure 6. Serum concentration (pg/ml) of anti-inflammatory interleukins of IL-4 and IL-10 derived from blood of animals in treatment groups (n=6 animals in each groups). EAE; experimental autoimmune encephalomyelitis, Dexe; dexamethasone, PBS; Phosphate-buffered saline, MPM2; macrophage M2. *** represented significant changes.

Figure 7