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Suppression of neuro inflammation in experimental autoimmune encephalomyelitis by glia maturation factor antibody

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Abstract

Glia maturation factor (GMF), a protein primarily localized in the central nervous system (CNS) was isolated, sequenced and cloned in our laboratory. We previously demonstrated that GMF mediates the experimental autoimmune encephalomyelitis (EAE)-induced production of proinflammatory cytokines and chemokines in the central nervous system of mice. In the present study we show that immunization with myelin oligodendrocyte glycoprotein peptide 35–55 (MOG35–55) caused an early onset (day 7–9 post immunization) and severe EAE with a mean peak score of 3.5 ± 0.5 in mice. Neutralization of GMF with four injections of anti-GMF antibody 5 to 11 days postimmunization delayed the time of onset (day 12–14 post immunization) and significantly reduced the severity of EAE (mean peak score of 1.5 ± 0.4). Consistent with these clinical scores, histological examination of the CNS of these mice revealed profound differences between GMF-antibody treated mice and isotype matched control-antibody treated mice. Histological analysis of the spinal cord and brain showed severe inflammation and demyelination in the control antibody-treated mice whereas significantly reduced inflammation and demyelination was detected in GMF-antibody-treated mice at day 8, 16, and 24 post immunization. The decreased incidence and reduced severity of EAE in GMF-antibody-treated mice was consistent with the significantly reduced expressions of proinflammatory cytokines and chemokines. Our overall results demonstrate that neutralization of endogenous GMF with an affinity purified GMF antibody significantly decreased the inflammation, severity and progression of immunization induced active, passive and relapsing-remitting EAE. Treatment of mice with isotype-matched control antibody did not have any effect on EAE. Taken together, these results demonstrate the critical role of GMF in EAE, and GMF antibody as a potent anti-inflammatory therapeutic agent for effectively suppressing EAE in mouse models of major types of multiple sclerosis (MS).

Keywords

Experimental autoimmune encephalomyelitis (EAE); Multiple sclerosis (MS); Glia maturation factor (GMF); Myelin oligodendrocyte glycoprotein 35–55 (MOG 35–55); Neuroinflammation; Cytokines/chemokines

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1. Introduction

Multiple sclerosis (MS) is a demyelinating disorder characterized by an autoimmune response to myelin antigens, resulting in widespread myelin destruction accompanied by damage to the underlying axon. This event is thought to be the result of a combined autoimmune response to some of the myelin components [4,18,41]. The pathogenesis of the disease is characterized by the activation of glial cells and infiltration of mononuclear cells, predominantly antigen-specific CD4+ and CD8+ T cells and B cells, in the central nervous system. The infiltrating mononuclear cells and the activated glial cells produce a variety of biological response modifiers, including deleterious free radicals, reactive oxygen species (ROS), reactive nitrogen species (RNS) and proinflammatory cytokines/chemokines, resulting in the demyelination of axons. High levels of pro inflammatory cytokines and chemokines (small chemotactic cytokines) in the brain are also thought to contribute to the initiation and maintenance of EAE [17,37]. The activated T cells, microglia and astrocytes produce a variety of proinflammatory molecules such as tumor necrosis factor- α (TNF- α), interferon- γ (IFN- γ), interleukin-1 beta (IL-1 β), IL-12, IL-23, and granulocyte macrophage-colony stimulating factor (GM-CSF). GM-CSF produced by activated astrocytes has a specific effect on the proliferation of microglia. Cytokines play a critical role in defining the Th1 or Th2 nature of the immune response and regulating inflammation in the CNS. Much of our current knowledge about contributing factors of MS is based on animal models of experimental autoimmune encephalomyelitis (EAE), such as: C57Bl/6/MOG35-55, SJL/PLP139-151 and adoptive transfer-EAE (AT-EAE). Research efforts in recent years on GMF, a highly conserved brain-specific protein, which was isolated, sequenced and cloned in our laboratory [21,24,46], have demonstrated an immunomodulatory function for GMF. Recently, it has been established that overexpression of GMF in astrocytes leads to immune activation of microglia through the secretion of granulocyte-macrophage-colony stimulating factor (GM-CSF) [52]. Moreover, on gene expression by DNA microarray analysis [49], we have found a significant increase in the expression of several genes, such as major histocompatibility complex (MHC) proteins, IL-1 β , MIP-1, all of which have been associated with the development of EAE. We also reported the stimulation of p38 MAP kinase pathway [22,47,48] and NF- κ B [25] by GMF in astrocytes. Based on GMF's ability to activate microglia and induce several well-established pro-inflammatory mediators, we hypothesize that intracellular GMF is involved in the pathogenesis of inflammatory demyelinating diseases of the central nervous system such as MS and EAE. Our recent experiments to test this hypothesis using GMF-deficient (GMF-KO) mice, which were developed in our laboratory [23], demonstrated a significant decrease in incidence, delay in onset, and reduced severity of EAE in GMF-knockout mice [53,55]. In the present study we investigate our novel therapeutic approach to effectively suppress GMF-function in EAE. Building on our recent success in suppressing GMF expression in vitro, we used an anti-GMF antibody to neutralize endogenous GMF protein in EAE mice. The central hypothesis is that the successful suppression of endogenous GMF-function will prove to be an effective and selective strategy to slow, and perhaps reverse, pathogenic processes in EAE.

2. Results

2.1. GMF-antibody treatment attenuates actively induced, passively transferred, and relapsing-remitting EAE

To examine the potential therapeutic action of GMF-antibody in EAE, GMF activity was blocked in mice by administration of a GMF-neutralizing antibody. In the actively induced EAE models, MOG35-55-immunization induced an early onset (7–9 days post immunizations) and severe EAE with a mean peak score of 3.5 ± 0.5 . Neutralization of GMF with four intravenous injections of GMF-antibody 5 to 11 days after the immunization delayed the time of onset (12–14 days) but significantly reduced the severity of EAE to a

mean severity score of 1.5 ± 0.4 . Where as, identical injections of an isotype-matched control antibody did not have any effect on the onset or course of EAE severity following MOG35-55-immunization (Figure 1A). This potent suppressing effect of the GMF-neutralizing antibody and failure of the control antibody to do so remained in effect throughout the experimental period. The GMF-antibody also strongly inhibited EAE induced by the adoptive transfer of encephalitogenic T-cells. Figure 1B shows that adoptively transferred EAE in control antibody treated mice reached a peak clinical score of 3.3 ± 0.75 , and then decreased to a score of 1.7 ± 0.35 and remained at this level for the rest of the experimental time. Identical injections of GMF-antibody significantly reduced the severity of the adoptively transferred EAE. The GMF-antibody reduced the peak mean clinical score to 1.4 ± 0.35 , and then decreased to a score of 0.5 and remained at this level. The results also show a delayed time of onset of the disease from 10 days in the control-antibody or no antibody treated mice to almost 14 days and decreased incidence of EAE in GMF-antibody treated mice. These results demonstrate that treatment with the GMF-antibody reduces the incidence and severity of both active and adoptively transfer EAE.

In relapsing-remitting EAE induced by PLP139-151-immunization in SJL/J mice, severe EAE was induced with three peaks of mean clinical scores of 3.5 ± 0.75 , 3.95 ± 0.4 , and 3.65 ± 0.5 at 16, 27, and 40 days post immunization in the control mice (no antibody and control-antibody treated). The scores remained elevated at 3.0 ± 0.5 . GMF-antibody injections significantly reduced the first two relapsing peak scores to 1.5 ± 0.4 , and almost eliminated the third relapse, mean clinical scores well below 1.0 (Figure 1C). Additionally, the results show that the GMF-antibody also effectively delayed the onset of EAE from 8–10 days in the control or no antibody treated mice to over 15 days in GMF-antibody treated mice. These results indicate that administration of GMF-antibody during ongoing EAE is effective in reducing the severity of relapses.

Thus, the overall results shown in Figure 1 clearly demonstrate that GMF-antibody treatment attenuates all three forms of EAE: actively induced, passively transferred, and relapsing-remitting EAE.

2.2. GMF-antibody-treated mice show reduced inflammation and demyelination

The histopathological scores for inflammation and demyelination in MOG35-55-induced EAE are illustrated in Figure 2. Both inflammation and demyelination scores were elevated in the immunized control-antibody treated mice. GMF-antibody treatment significantly reduced the inflammation scores from 50.5 ± 6.8 to 14.5 ± 4.0 , from 60 ± 5.5 to 24.0 ± 4.0 , and from 30.5 ± 6.5 to 9.5 ± 1.5 at 8, 16 and 24 days post-immunization, respectively. GMF-antibody also reduced the demyelination scores at 8, 16, and 24 days post immunization samples from 18.5 ± 3.0 to 5.0 ± 1.0 , from 40.8 ± 5.5 to 7.5 ± 2.5 , and from 22.8 ± 3.5 to 5.0 ± 1.5 , respectively. These reductions in inflammation and demyelination scores by GMF-antibody injections were highly significant.

2.3. GMF-antibody treated mice fail to sustain inflammation in the CNS of immunized mice

The effects of GMF-antibody treatment on the inflammation of the CNS were examined by histological studies of the fixed spinal cord (Figure 3A) and brain (Figure 3B), tissues were sampled at 8, 16 and 24 days post immunization using haematoxylin eosin staining. At day 16, the time at which the disease in wild type mice peaked, there were inflammatory infiltrating cells in both control-antibody treated and GMF-antibody treated mice. However, control-antibody treated mice showed numerous infiltrating cells scattered throughout the white matter parenchyma, while GMF-antibody treated mice exhibited significantly reduced infiltration of these inflammatory cells in the spinal cord, (Figure 3A) as well as in the brain (Figure 3B). At day 24, there was little change in the number of inflammatory infiltrates

within the CNS of control-antibody treated mice (Fig. 3 A and 3B); however, there was a slight increase in cellular infiltration into the spinal cord, (Figure 3A) compared with that observed at day 16 in GMF-antibody treated mice.

Transverse sections of cervical, upper thoracic, lower thoracic, and lumbar regions of the spinal cord of mice injected with GMF-antibody, control-antibody, and no antibody injected mice were compared at day 8 and 16 post immunizations were stained with Luxol Fast Blue to visualize demyelination (Figure 3C). The results show that control-antibody or no antibody-treated EAE mice developed extensive myelin loss (demyelination) in the spinal cord. In contrast, the mice treated with GMF-antibody showed a significant decrease in demyelination both at day 8 and 16 post immunizations.

These results demonstrate that GMF-antibody treatment reduces inflammation and demyelination in MOG-induced EAE.

2.4. GMF-antibody-treated mice exhibit decreased expression of proinflammatory cytokines, chemokines, and iNOS in the CNS

We examined mRNA levels of the cytokines TNF- α , IL-1 β , IL-6, IFN- γ , and the chemokines GM-CSF, MIP-1, MIP-2, MCP-1, IP-10, and iNOS by quantitative real-time RT-PCR in the spinal cord and brain of EAE mice (Figure 4). The levels of proinflammatory cytokines/chemokines known to be involved in the pathogenesis of EAE were significantly decreased in the CNS of GMF-antibody treated mice compared with control-antibody treated mice. In order to confirm whether the detection of mRNA by real-time RT-PCR correlated with protein expression, ELISA analysis was conducted. The protein levels of cytokines and chemokines in the spinal cords of mice at 8, 16, and 24 days post MOG immunization, are shown in Figure 5, respectively. There is a distinct pattern of very large increases in the levels of IFN- γ , TNF- α , MCP-1 and GM-CSF in the spinal cord at about the time of peak severity in control immunized (no antibody and control-antibody treated) mice. GMF-antibody treatment consistently caused a drastic reduction of proinflammatory mediators to significantly low levels at all three sampling times.

3. DISCUSSION

We previously described the inflammatory role of GMF in the CNS. This highly conserved CNS protein mediates the downstream induction of GM-CSF through p38 MAPK/NF κ B signaling. This leads to the excessive production of proinflammatory cytokines/chemokines and iNOS in mixed cultures of brain cells, resulting in the death of oligodendroglial and neuronal cells [52]. Although the etiology of MS is not fully understood, CD4⁺ myelin-reactive Th1 cells are believed to be responsible for the induction and progression of MS and EAE. During active MS and EAE, myelin-reactive antibodies are also generated. Using GMF-knockout mice, we have previously shown that MOG-induced EAE was muted, T cell differentiation into Th1 cells was suppressed, and Th2 cell numbers were increased [50,53]. Additionally, several EAE associated inflammatory events were suppressed and the circulating anti-MOG antibody was also diminished in immunized GMF-KO mice [50]. Additionally, we also reported earlier that silencing the over production of GMF directly by GMF specific siRNA remarkably prevented death of oligodendroglial and neuronal cells along with suppression of excessive proinflammatory cytokines/chemokines production in a mixed culture of brain cells [50]. Thus, the two main processes, CD4-myelin Th1 cells and the inflammation-mediated death of oligodendrocytes, involved in the progression of the EAE are suppressed in the absence of GMF or when GMF is selectively silenced. Neutralization of GMF with antibody is expected to do the same.

During active MS and EAE, myelin-reactive antibodies are generated in addition to significantly high amounts of destructive cytokines. Of these cytokines, IFN- γ and TNF- α have been shown in particular to elicit considerable damage to oligodendrocytes, and are present in significantly high levels in the MS brain [34,35]. TNF- α elicits its effect by activating c-jun aminoterminal kinase-3 (JNK-3), leading to apoptosis [20] and inhibition of JNK-3 protects the cells from undergoing apoptosis [14,43]. On the other hand, exposure of IFN- γ to oligodendrocytes disrupted protein secretory pathways, leading to endoplasmic reticulum (ER) stress and increased levels of phosphorylated eIF2- α . As a result of ER stress, caspase 12 is activated this in turn activates caspase 3 leading to apoptosis. Moreover, ectopic expression of IFN- γ induces hypomyelination in the developing CNS [11]. These events lead to apoptosis of oligodendrocytes. In fact, treatment of MS patients with IFN- γ leads to exacerbation of the disease [33]. However, it has been shown that matured oligodendrocytes are less sensitive than progenitors to IFN- γ effect [1,2,34]. Thus, among the different cytokines secreted by T cells, both IFN- γ and TNF- α plays a major role in the death of oligodendrocytes. We previously described the inflammatory role of GMF in CNS. This highly conserved CNS protein mediates the downstream induction of GM-CSF, through p38 MAPK / NF κ B signaling, leading to excessive production of proinflammatory cytokines, chemokines, and iNOS in mixed cultures of brain cells and death to oligodendroglial and neuronal cells [52].

Reactive oxygen and nitrogen species (ROS & RNS) are known to damage lipids, proteins and nucleic acids in a variety of cells and in oligodendrocytes resulting in demyelination and tissue damage [30,40]. Increased CNS levels of ROS and RNS were found in MS and EAE. The role of nitric oxide (NO) is found to be microenvironment and concentration dependent [3]. NO is also found to increase permeability of blood-brain barrier, though the exact mechanism is not clear. Recently, It has been shown [13] that the induction of the enzyme inducible nitric oxide synthase (iNOS), by myelin basic protein-primed T cells and in microglia, was responsible for the inflammation in CNS. It has been shown that TNF- α and IL-1 β exert neurotoxicity in concert with up regulation of iNOS and contributes to neuro-protection and plasticity in the absence of iNOS [42]. Amelioration of EAE by inhibitors of iNOS and antisense oligonucleotides against iNOS suggests an important role for iNOS-derived NO in the pathogenesis of EAE [12,16].

In the present report we demonstrate attenuation of immunization induced severe EAE, inflammation, demyelination and the production of GM-CSF and other proinflammatory molecules including iNOS in spinal cords of mice by an affinity purified GMF neutralizing GMF-antibody. The effectiveness of GMF-Ab remained strong in all of the three, active, passive and relapsing-remitting models of EAE. We used i.v. injections of antibodies during 5 to 11 days after immunization instead of intracerebral / intrathecal injections since, the CNS penetration of i.v. injected antibodies is allowed through disrupted blood brain barrier early in immunization induced EAE. For histological and proinflammatory molecules analysis, spinal cords were sampled on days 8, 16, and 24 post immunization corresponding to the onset, at peak and post peak regression of EAE severity, respectively. At all the three post immunization times, GMF-antibody reduced infiltrating inflammatory cells, significantly suppressed EAE severity, associated proinflammatory cytokines/chemokines, iNOS, and significantly reduced the spinal cord demyelination. These effects of GMF-antibody were expected and paralleled to our previous report of immunization with MOG in GMF-deficient (GMF-KO) mice that were resistant to EAE [50,53]. The deficiency of GMF in GMF-KO mice had no effect on MOG 35–55 induced proliferation of CD4⁺ T cells but the circulating anti-MOG antibody levels were significantly low in immunized mice. Moreover, in GMF-KO mice following MOG-immunization, we observed prevention of T cells differentiation in to Th1 cells, reduced number of activated glial cells (astrocytes and microglia), and reduced levels of GM-CSF along with other inflammatory mediators

including cytokines, chemokines and iNOS [50]. The report that GM-CSF neutralizing antibody prevents active, passive and relapsing- remitting EAE is consistent with our observation that GMF mediates the immunization induced EAE since GM-CSF functions down stream of GMF. We also reported that GMF is required for activation of GSK-3 β [54], a well known inhibitor of nuclear factor E2-related factor 2 (Nrf2) that protects neurons from ROS cytotoxicity in vitro and in vivo [8,39]. Therefore, it is reasonable to expect that neutralization of GMF could prevent inhibition of Nrf2 from GSK-3 β and allow an extra layer of neuroprotection.

In recent years several efforts were made to use monoclonal antibodies for blocking specific target of interest in the pathogenesis of MS as a therapeutic option. In this regard, while natalizumab received FDA approval in US and by the European Medicines Agency, others such as rituximab, alemtuzumab, and daclizumab are still in clinical trials. Natalizumab, a humanized monoclonal antibody, targets the very late antigen-4 (VLA-4) which has a crucial role in the transmigration of immune cells across the blood brain barrier [36,38]. Natalizumab is also approved for use in Crohn's disease. Other monoclonal antibodies that are in clinical trials include Rituximab, a human-mouse chimeric mAb directed against the CD20 antigen and Alemtuzumab, a humanized derivative of the rat mAb directed against the CD52 antigen. Treatment with rituximab or alemtuzumab result in depletion of target antigen by complement mediated cytotoxicity, antibody-dependent cytotoxicity and apoptosis [44]. Rituximab and alemtuzumab are currently FDA approved for non-Hodgkin's lymphoma and refractory chronic lymphocytic leukemia, respectively [15,27]. Depletion of circulating T-cells has been a strategy to treat MS. But such strategies including the modifiers of lymphocyte migration have some serious and fetal side effects irrespective of their efficacy in clinical trials [10,28]. On the other hand, intervention with GMF neutralizing anti-body or GMF specific siRNA could achieve full spectrum suppression of pathology and physiologies of EAE and are not expected to share fetal side effect of inhibitors of immune cell-migration due to different mechanism of action. The absence of GMF does not prevent antigen stimulated proliferation of T cells and GMF-KO mice do not exhibit any abnormality or susceptibility to opportunistic pathogens (unpublished observation). The presence of soluble Nogo-A was reported in CSF of MS patients Nogo-A, a major component of CNS myelin, is known to inhibit the regeneration of damaged axons [19]. Silencing / neutralization of Nogo-A or LINGO1 appear to promote remyelination in EAE [29], therefore, silencing/neutralizing GMF combined with antagonists of LINGO1 will be an effective strategy to treat EAE/MS disease.

SUMMARY

Various antibodies have been used to treat MS in clinical trials with variable success. We did not find any obvious abnormalities or side effects following GMF-antibody treatments. The GMF- antibody is also effective against active, passive, and relapsing-remitting EAE. Thus, our overall results demonstrate that immunization-induced EAE is dependent on GMF mediated events that causes the excessive production of several proinflammatory cytokines/chemokines. This supports the idea that targeting GMF with a specific antibody can provide a novel, rational, effective, and non-toxic therapeutic option for intervention in MS/EAE.

4. Experimental Procedures

4.1. Reagents

Myelin oligodendrocyte glycoprotein peptide 35–55 (MOG35-55) and pertussis toxin were purchased from Sigma-Aldrich, St. Louis, MO. Complete Freund's adjuvant (FCA) was from Difco, Detroit, MI. G2-09-N07 was a monoclonal antibody against GMF and was affinity-purified with protein-G plus. ELISA kits for mouse TNF- α (Cat # KMC3011), IFN-

γ (Cat # KMC4021), GM-CSF (Cat # KMC2011) and MCP-1 (monocyte chemoattractant protein-1, Cat # KMC0061) were obtained from Biosource International, CA. PCR primers were synthesized at Integrated DNA Technologies (Coralville, IA). ThermoScript™ RT-PCR system (Cat #11146-016) for first-strand cDNA synthesis was purchased from Invitrogen Corporation, Carlsbad, CA. RNazol B reagent was from Tel-Test, Inc., Friendswood, TX.

4.2. Induction of EAE

C57BL/6 and SJL/J mice were purchased from Harlan Sprague Dawley, Inc., Indianapolis, IN, and maintained in the animal colony at the University of Iowa and were used in accordance with the guidelines approved by the IACUC and National Institutes of Health. For active induction of EAE, C57BL/6, 8–10 week-old, female mice were immunized with subcutaneous injection of 150 μ g myelin oligodendrocyte glycoprotein peptide 35–55 (MOG 35–55) in 100 μ l PBS and mixed with 100 μ l of complete Freund's adjuvant (CFA) and boosted day 0 and day 2 with i.p. injections of 300 ng pertussis toxin. Control mice received identical injections without MOG 35–55 and were boosted with the toxin. The mice were weighed and scored daily in a double blinded fashion according to the scoring scale of 0 to 5, score 0, no disease; score 1, tail weakness; score 2, weakness in hind limb; score 3, complete hind limb paralysis; score 4, hind limb paralysis with fore limb weakness or paralysis; and score 5, moribund or deceased. For passively transferred EAE, 10 days after immunized with MOG 35–55, CD4+ T cells were isolated from spleens of a group of donor mice using a commercial CD4+ T cell isolation kit (Miltenyi Biotech, Auburn, CA) according to the manufacturer's instructions. The isolated CD4+ T cells were further stimulated with MOG 53–55 (20 μ g/ml) for 72 h in tissue culture conditions, washed in excess of PBS and then 10^7 cells were injected in each of the recipient mice. The mice were weighed and scored daily as described above. Relapsing-remitting EAE were induced in SJL/J mice (female) with PLP139-151, 2mg/ml /CFA 1:1. Relapses are scored as follows: a relapse is counted if the mouse shows a reduction in score of at least one point for 2 or more days, followed by an increase in score of at least a point for two or more days. The mean clinical scores were calculated by adding every day clinical score for all mice in a group and then dividing by the total number of mice. Onset of EAE was defined as the day of clinical score of one, and peak as maximum severity of clinical signs after immunization with encephalitogenic peptide. All the animals were euthanized at the end of each experiment.

4.3. Antibody treatment

We examined the inhibitory effect of highly purified, specific and well characterize GMF-neutralizing antibody on three models of EAE, active, passive, and relapsing-remitting EAE. The antibody used was an affinity purified G2-09-N07 antibody, generated and characterize [45,46,51–53,56] in our laboratory, against GMF that cross-react with human, rat and mouse GMF protein. We showed earlier using this antibody [51,56] a lack of GMF signal in GMF-KO mice. With respect to the GMF-Ab dose, we have predetermined (data not shown) optimal preventive dose and used 2mg/kg/mouse as an optimal treatment dose. The first (loading) dose (2 mg/kg/mouse) of GMF-antibody was administered by intravenous injection at day 5 post immunization, a time at which the brain-blood barrier is breached, followed by every second day (day 7, 9, and 11). GMF-antibody-treated group was compared with isotype-matched mouse IgG (2 mg/kg/mouse) treated and no antibody-treated groups, within the same experiment. Each group consisted of 12–16 mice.

4.4. Histological assessment and immunohistochemistry

At three time points, determined by clinical observation corresponding to the onset (8 days), peak of the disease (16 days), and following recovery (24 days), six mice in each experimental group were anesthetized by intraperitoneal injection of sodium pentobarbital

and transcardially perfused with PBS and by 4% paraformaldehyde in phosphate buffer. Brain and spinal cord were removed, fixed and embedded. The sections were stained with hematoxylin and eosin to reveal infiltrating inflammatory cells and with Luxol fast blue for the evidence of demyelination and scored under microscope, essentially as described earlier [5–7,31,32,53] [5,6,53]. In brief, five micrometer thick transverse sections (five sections per mouse) were taken from cervical, upper thoracic, lower thoracic, and lumbar regions of spinal cord. The sections were stained with hematoxylin and eosin to reveal infiltrating inflammatory cells and with Luxol fast blue for the evidence of demyelination. Each spinal cord section further subdivided into anterior, posterior and two lateral columns (4 quadrants). The signs of inflammation and demyelination in the spinal cord sections were scored under microscope in a blinded manner by two examiners. Each quadrant showing the infiltration of cells was assigned a score of one inflammation and the quadrant that showed perivascular lesion and loss of myelin staining a score of one demyelination. The pathologic score (inflammation or demyelination) for each group was expressed as the percentage positive over the total number of quadrants examined.

4.5. Quantitative estimation of mRNA by real-time RT-PCR

Total RNA was extracted from spinal cord and brain tissues of mice (6 mice per group) during the active EAE at day 16 post immunization with encephalitogenic MOG 35–55, by the acid guanidinium thiocyanate-phenol-chloroform method, using the RNeasy RNeasy spin kit, in a reaction volume of 20 μ l containing 1 μ g of total RNA, 100 ng random hexamer, 40 units RNaseOut, and 4 μ l of a solution comprising 250 mM Tris acetate, pH 8.4, 375 mM potassium acetate, 40 mM MgCl₂, 5 mM dithiothreitol, 1 mM dNTP mixture and 15 units of ThermoScript reverse transcriptase enzyme. Transcription was carried out at 24°C for 10 min and 50°C for 4 h. After stopping the reaction by incubation at 90°C for 15 min, 5% of the RT mixture was mixed with 2 $\times$ SYBR green PCR master mix (Applied Biosystems) and gene-specific primers in a final volume of 20 μ l. The primers were designed with Primer Express software. The primer pairs were selected to yield a single amplicon based on dissociation curves and analysis by acrylamide gel electrophoresis. Real-time quantitative PCR was performed in a model ABI Prism 7000 sequence detection system. The thermal cycler parameters were as follows: hold for 2 min at 50°C and 10 min at 95°C for one cycle followed by amplification of cDNA for 40 cycles with melting for 15 s at 95°C and annealing and extension for 1 min at 60°C. The values were normalized using 18S rRNA as an endogenous internal standard. The relative concentration of mRNA was calculated using the formula $\times = 2^{-\Delta\Delta C_t}$, where $\times$ = fold difference in amount of starting material between two treatment groups (experimental versus control, here KO versus Wt), $\Delta\Delta C_t = \Delta E - \Delta C$, and $\Delta E = C_{t_{exp}} - C_{t_{18Sexp}}$, $\Delta C = C_{t_{control}} - C_{t_{18Scontrol}}$. “Ct” stands for threshold cycle which is the fractional cycle number at which the amount of amplified target reaches a fixed threshold [26]. The sequences of the mouse primers used for each gene is given below:

TNF- α : F) CATCTTCTCAAAATTCGAGTGACAA
R) TGGGAGTAGACAAGGTACAACCC

IL-1 β : F) CAACCAACAAGTGATATTCTCCATG
R) GATCCACACTCTCCAGCTGCA

IL-6: F) GAGGATACCACTCCCAACAGACC
R) AAGTGCATCATCGTTGTTCATACA

IFN- γ : F) TCAAGTGGCATAGATGTGGAAGAA
R) TGGCTCTGCAGGATTTTCATG

GM-CSF: F) GCCATCAAAGAAGCCCTGAA
R) GCGGGTCTGCACACATGTTA

MCP-1: F) CTTCTGGGCCTGCTGTTCA
R) CCAGCCTACTCATTGGGATCA

MIP-1: F) CTCCCACTTCCTGCTGTTTC
R) GGGAGACACGCGTCCTATAA

MIP-2: F) GAGCTTGAGTGTGACGCCCCCAGG
R) GTTAGCCTTGCCCTTTGTTTCAGTATC

IP-10: F) GCCGTCATTTCTGCCTCAT
R) GCTTCCCTATGGCCCTCATT

iNOS: F) CAGCTGGGCTGTACAAACCTT
R) CATTGGAAGTGAAGCGTTTCG

18S: F) TTGACGGAAGGGCACCACCG
R) GCACCACCACCCACGGAATCG

4.6. Enzyme-linked immunosorbent assay (ELISA)

Whole spinal cord tissue of mice (6 mice per group) at 8, 16, and 24 days post MOG 35–55 immunization (active EAE) were homogenized and the cytokine/chemokine concentrations were estimated by sandwich immuno-assay procedure as specified in the manufacturer's protocol. Briefly, to 96-well microtiter ELISA plates pre-coated with anti-cytokine capture antibodies, the cytokine standard and samples were added and incubated overnight at 4°C followed by washing. Corresponding biotinylated antibodies, horseradish peroxidase-conjugated streptavidin and TMB substrate used to develop a yellow color and read by a Microplate reader at 450 nm. The concentration of cytokine/chemokine was estimated from a standard curve generated with each run. The lower detection limits of these ELISA are in the range of 10–15 pg/ml. ELISA data are presented as mean values $\pm$ standard deviations and represent at least two independent experiments with similar results.

4.7. Statistical analysis

Significant differences in onset of EAE were analyzed by ANOVA test and differences in EAE clinical score by Mann-Whitney test. All the experiments were performed three to five times to permit statistical analysis. Statistical significance was established at $p < 0.05$.

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Figure 1.

Administration of GMF-antibody significantly suppresses EAE in immunized mice. **(A)** Actively induced EAE, mice (C57BL/6) were injected four times i.v., from day 5 to day 11 (every second day beginning day 5) postimmunization, with GMF-antibody or isotype matched control-antibody. Arrow indicates treatment days, 5, 7, 9, and 11 postimmunization. Data shown represents three independent experiments. **(B)** adoptively transferred EAE, EAE was induced by adoptive transfer of encephalitogenic T cells in C57BL/6 mice. Starting on day 5 post transfer and every second day thereafter, mice received GMF-antibody or isotype matched control-antibody. Data shown represents two independent experiments; **(C)** relapsing-remitting EAE, RR- EAE was induced in SJL mice

by immunization with PLP139-151. Mice were treated on day 5, 7, 9, and 11 with GMF-antibody or isotype matched control- antibody. Data shown represents two independent experiments. The differences in clinical scores between GMF-Ab treated mice and control-Ab or no antibody treated mice are statistically significant (* $p < 0.01$).

Figure 2. Histopathological scores for the inflammation and demyelination in MOG-induced active EAE. Mice were injected with GMF-antibody or isotype matched control-antibody, every second day beginning day 5 after immunization with MOG35-55. Differences between GMF-antibody and control-antibody treated mice were highly significant ($p < 0.01$) at all the time points examined (day 8, 16, 24 postimmunization).

Figure 3.

GMF-antibody treatment significantly reduces the inflammation in MOG-induced EAE. Histological analysis (H & E staining) showing significantly reduced inflammatory infiltrations in the spinal cord (A) and (B) brain of mice injected with GMF-antibody compared to control-antibody injected mice at day 8, 16, 24 post immunizations. (C) Transverse sections of cervical, upper thoracic, lower thoracic and lumbar regions of spinal cord of mice injected with GMF-antibody compared to control-antibody injected mice at day 8 and 16 post immunizations were also stained with Luxol Fast Blue, as described in Experimental Procedures. The photomicrographs are representative sections from spinal cord and brain of control-antibody and GMF-antibody treated mice. Magnifications: 10X, 20X, 40X

Figure 4.

Cytokine/chemokine mRNA levels in the spinal cords and the brains at day 16 post MOG₃₅₋₅₅-immunized wild-type mice with active EAE (black bar), MOG₃₅₋₅₅-immunized wild-type mice treated with an isotype-matched control-antibody (red bar), and MOG₃₅₋₅₅-immunized wild-type mice treated with GMF neutralizing antibody (green bar) were determined by quantitative real-time RT-PCR. Data represent the mean $\pm$ S.D. from three independent experiments and the results are expressed as the relative mRNA level. (* p < 0.01).

Figure 5.

Suppression of pro inflammatory cytokines/chemokines in the CNS of MOG-induced EAE mice treated with GMF-antibody or isotype-matched control-antibody. IFN- γ , TNF- α , MCP-1 and GM-CSF levels in spinal cord homogenates were determined by ELISA in active EAE mice on days 8, 16, and 24 post immunizations with MOG 35–55. Data represent the mean $\pm$ S.D. from three independent experiments. Cytokines-chemokines levels were quantitated by comparison to the standard curves generated using corresponding recombinant standards. (* $p < 0.01$)

Abbreviations: CFA, complete Freund's adjuvant; MOG, myelin oligodendrocyte glycoprotein peptide 35–55; C-Ab, control antibody.