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DIMINISHED CYTOKINE AND CHEMOKINE EXPRESSION IN THE CENTRAL NERVOUS SYSTEM OF GMF-DEFICIENT MICE WITH EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

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Abstract

Pro-inflammatory cytokines/chemokines are implemented in the pathogenesis of experimental autoimmune encephalomyelitis (EAE), an animal model with clinical and pathological similarities to multiple sclerosis. We have previously shown that over-expression of glia maturation factor (GMF) in glial cells cause excessive production and secretion of pro-inflammatory cytokines/chemokines sufficient to destroy the myelin forming oligodendroglial cell *in-vitro*. In this present investigation, we evaluate the expression of pro-inflammatory mediators in the central nervous system (CNS) of GMF+/+ (wild type) mice and GMF-/- (GMF-knockout) mice at the peak of EAE induced by immunization with MOG 35–55 peptide. GMF+/+ (Wt) mice developed severe EAE with a maximal mean clinical score of 3.6 ± 0.5 by day 16 post immunization, whereas GMF-KO mice showed significantly delayed EAE with an average onset on day 26 pi with reduced mean clinical score of 1.3 ± 0.3. Three of fifteen Wt mice as compared to none of GMF-KO mice died of EAE. Encephalitogenic cells from Wt mice transferred to recipient GMF-KO mice caused very mild and with low incidence of EAE. We determined the differences in the expression of cytokines, IFN- γ , TNF- α , IL-1 β , IL-6, IL-4, IL-10, and chemokines, MIP-1, MIP-2, IP-10, MCP-1, GM-CSF mRNA by quantitative real time RT-PCR in brain and spinal cord. Our results demonstrate significantly low levels of pro inflammatory cytokines/chemokines in the CNS of GMF-KO mice and increased expression in Wt mice with EAE. Our data suggest that GMF play a critical role in CNS inflammation.

Keywords

Glia maturation factor (GMF); Cytokines/Chemokines; Inflammation; Experimental autoimmune encephalomyelitis (EAE); Multiple sclerosis (MS); Myelin oligodendrocyte glycoprotein (MOG)

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1. Introduction

Multiple sclerosis (MS) is a chronic, relapsing-remitting inflammatory demyelinating disease of central nervous system. The pathogenesis of the disease is characterized by the activation and infiltration of mononuclear cells, predominantly antigen-specific CD4⁺ and CD8⁺ T cells and B cells, in central nervous system, reactivation by resident antigen-presenting microglial cells, secretion of proinflammatory cytokines/chemokines along with generation of other inflammatory mediators, such as complement, highly reactive free radicals (ROS, RNS), resulting in the demyelination of axons (Genain and Hauser, 2001; Iglesias et al., 2001; Noseworthy et al., 2000). Experimental autoimmune encephalomyelitis (EAE) is an animal model of MS, produced in laboratory animals by immunization with myelin-derived antigens. The disease is induced in susceptible mice, rat, or nonhuman primates by immunization with myelin basic protein (MBP), proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG) or by the transfer of autoreactive CD4⁺ T-cells (Lington et al., 1993; Tuohy, 1994). The disease EAE progresses by infiltration of autoreactive, myelin-specific T cells and activated macrophages into the CNS. These events start a series of inflammatory reactions in addition to direct toxicity of infiltrating cells and antibody-mediated toxicity. Cytokines and chemokines play an important role in the initiation and maintenance of the inflammatory environment in EAE. Precise mechanisms of action of these inflammatory mediators remain unclear.

Recent studies on glia maturation factor (GMF), a brain-specific protein, isolated, sequenced and cloned in our laboratory (Kaplan et al., 1991; Lim et al., 1989; Lim et al., 1990), have given new insights into its role in immunomodulatory and proinflammatory functions in CNS (Lim and Zaheer, 1996; Lim et al., 2000; Zaheer et al., 2002; Zaheer et al., 2004; Zaheer et al., 2006; Zaheer et al., 2007). We have established that overexpression of GMF in astrocytes leads to immune activation of microglia, through secretion of granulocyte-macrophage-colony stimulating factor (GM-CSF), and resulting in production of several pro inflammatory mediators that destroy oligodendrocytes, the myelin forming cells in CNS. We hypothesize that intracellular GMF is involved in the pathogenesis of inflammatory diseases of the central nervous system such as MS and EAE. More recently, we have shown that GMF ^{-/-} mice (GMF-KO) were resistant to EAE induced by active immunization with MOG-35–55 antigen, whereas GMF ^{+/+} (Wt) mice developed the disease characterized by significantly increased inflammation and demyelination in the central nervous system (Zaheer et al., 2007). In the present study, to analyze the inflammatory and immune mechanisms underlying the beneficiary effects of GMF deficiency in mice, we compared the development of EAE in wild type (Wt) and GMF-KO mice in both active and adoptive transfer models. To test our hypothesis that GMF exerts its effects by modulating the cytokine/chemokine profile in the CNS microenvironment, we studied the expression profile of cytokines, chemokines and inducible nitric oxide synthase (iNOS) during the progression of EAE in brain and spinal cord of GMF-KO and Wt mice.

2. Results

2.1. GMF-KO mice are relatively resistant to MOG-induced EAE in active and passive transfer models

GMF-dependent expression of several pro-inflammatory cytokines, such as TNF- α , IL-1 β , IL-6, GM-CSF and IP10, are responsible for the destruction of myelin-forming oligodendrocytes in the central nervous system (Zaheer et al., 2006b). In the present study we investigated the involvement of GMF in both, the induction and the effector phases of EAE. For this purpose, we immunized GMF-deficient (GMF-KO) and control wild type (Wt) mice (8–10 weeks-old female) with MOG 35–55 peptide, and followed EAE development. Results in Fig. 1A show that all fifteen Wt mice developed EAE within 16 days with a maximal mean

clinical score of 3.6 ± 0.5 . In the GMF-KO group only four mice out of sixteen developed EAE with a maximal mean clinical score of 1.3 ± 0.3 in 26 days. There was no mortality in GMF-KO mice whereas Wt mice had mortality rate of approximately 20% (3/15). The results show a significant decrease in incidence, delay in onset, and reduced severity of EAE in GMF-KO mice. These results demonstrated that GMF play a crucial role in the pathogenesis of EAE.

In order to determine if GMF plays a role in the induction phase, the effector phase, or in both the phases of EAE, we performed adoptive transfer experiments by intravenous injection of CD4⁺ T cells from MOG 35–55 immunized donor Wt and GMF-KO mice to recipient Wt and GMF-KO mice, respectively. Results in Fig. 1B show that encephalitogenic cells from Wt mice induced less severe and with a significant decrease in incidence of EAE in GMF-KO (3/8) when compared with the Wt mice (8/8). These results indicate that GMF plays an important role in the progression of EAE disease. Transfer of CD4⁺ T cells from MOG 35–55 immunized GMF-KO mice to recipient Wt or GMF-KO mice, respectively, induced significantly less severe EAE with delayed onset in both (2/8) Wt and GMF-KO recipient mice. Collectively, our results show that GMF plays an important role in both, the induction and the effector phases of EAE.

2.2. Reduced production of MOG 35–55-specific Th 1 cytokines and anti- MOG 35–55 IgG in GMF-KO mice

Next, we examined the T cell proliferative and cytokine responses. For this purpose, GMF-KO and Wt mice were immunized with MOG 35–55 followed by isolation of CD4⁺ T cells ten days later as described in Methods. Results in Fig. 2A show a dose-dependent proliferation of T cells in response to MOG 35–55 in both GMF-KO and Wt mice. These results suggest that absence of GMF did not change MOG-specific T cell proliferation. Results in Fig. 2B show a significant reduction in the production of cytokines associated with Th1 response, such as IFN- γ and TNF- α , in GMF-KO CD4⁺ T cells exposed to MOG 35–55 (10 μ g/ml) when compared with the response in Wt cells. In contrast, levels of IL-4 and IL-10, cytokines associated with Th2 response, remained unchanged and at very low levels in both Wt and GMF-KO CD4⁺ T cells. Results in Fig. 2C show significantly lower levels of anti-MOG 35–55 IgG in the serum of GMF-KO when compared with Wt mice at the peak of the disease on day 16 and day 26 post immunization (day 16 pi in Wt and day 26 pi in GMF-KO mice, Fig. 1A). These results suggest that the resistance to MOG-induced EAE in GMF-KO mice could be due to a defective production of Th1 type pro inflammatory cytokines accompanied by marked impairment in the production of autoantibodies to MOG 35–55. Results in Fig. 3 show serum concentrations of IFN- γ , TNF- α , IL-4 and IL-10 in GMF-KO and Wt mice on day 16 and day 26 post immunized with MOG 35–55 as determined by ELISA. Significantly reduced serum concentrations of pro inflammatory cytokines, IFN- γ and TNF- α , were found in GMF-KO mice compared to the control Wt mice at day 16 pi. There were no significant differences in the levels of serum IL-4 and IL-10 between Wt and GMF-KO mice at day 16 and day 26 pi, and remained at low levels.

2.3. Reduced mRNA expression of pro inflammatory mediators in the CNS of GMF-KO mice with EAE

We analyzed the expression of inflammatory cytokines, chemokines, and iNOS mRNA in brain and spinal cord was analyzed by quantitative real time RT-PCR (Fig. 4A). We found a up-regulation of IFN- γ mRNA (13.8 ± 2.2 -fold), TNF- α mRNA (4.6 ± 1.1 -fold), IL-1 β mRNA (2.5 ± 0.5 -fold), and IL-6 mRNA (4.5 ± 1.0 -fold) at the peak of EAE disease (day 16 pi, mean score 3.6 ± 0.5) in the brain of Wt mice immunized with MOG 35–55 in CFA when compared with control Wt mice receiving CFA alone (Fig. 4A). Expressions of IL-4 and IL-10 mRNA were not significantly changed. The up regulation of chemokines, macrophage inflammatory protein -1 and -2 (MIP-1 and -2), monocyte chemoattractant protein-1 (MCP-1), interferon-induced

protein (IP)-10, and granulocyte-macrophage-colony stimulating factor (GM-CSF) have been shown to perpetuate the inflammatory environment with macrophage and leukocyte recruitment. Therefore, we examined the expression of various chemokine genes in the CNS of EAE mice. Results in Fig. 4B show a significant increase in the mRNA levels of MIP-2 (3.0 ± 0.4 -fold), MCP-1 (5.2 ± 1.1 -fold), IP-10 (4.5 ± 0.8 -fold), and GM-CSF (6.1 ± 1.1 -fold) at the peak of EAE disease in Wt mice immunized with MOG 35–55 as compared to CFA-injected Wt controls. In contrast, MOG 35–55 immunization in GMF-KO mice caused significant increased brain-levels of only IFN- γ mRNA (2.7 ± 0.3 -fold) and TNF- α mRNA (2.1 ± 0.4 -fold) at the peak of EAE disease in these mice (day 26pi, mean score 1.3 ± 0.3) when compared to non-immunized GMF-KO control mice injected with CFA without the MOG (Fig. 4C). Expression levels of other cytokines, IL-1 β , IL-6, IL-4, IL-10, and chemokines, MIP-1, MIP-2, MCP-1, IP-10, GM-CSF (Fig. 4D), mRNA did not differ significantly in brains of GMF-KO mice with or without MOG 35–55 immunization. Next, we examined mRNA expressions of cytokine and chemokine in spinal cord tissue. Significantly higher mRNA levels of cytokines (Fig. 4E) (IFN- γ mRNA 5.1 ± 2.1 -fold, TNF- α mRNA 30.5 ± 8.5 -fold, IL-1 β mRNA 15.1 ± 3.0 -fold, and IL-6 mRNA 55.0 ± 7.5 -fold) and chemokines (Fig. 4F), (MIP-1 mRNA 2.4 ± 0.3 , MIP-2 mRNA 5.5 ± 1.7 , MCP-1 mRNA 21.6 ± 5.3 , IP-10 mRNA 17.3 ± 4.2 , GM-CSF mRNA 62.9 ± 10.4) were found in the spinal cords of Wt mice at the peak of MOG-induced EAE. There were no significant differences in the levels of IL-4 and IL-10 mRNA. As seen in Fig. 4, G and H, GMF-KO mice at the peak of MOG-induced EAE (day 26pi, mean score 1.3 ± 0.3) had significantly lower levels of IFN- γ mRNA (1.4 ± 0.2 -fold), TNF- α mRNA (1.7 ± 0.4 -fold), IL-1 β mRNA (2.5 ± 0.3 -fold), and IL-6 mRNA (2.8 ± 0.5 -fold), IL-4 mRNA (1.3 ± 0.4 -fold), IL-10 mRNA (1.2 ± 0.4 -fold), MCP-1 mRNA (1.4 ± 0.1), IP-10 mRNA (1.5 ± 0.1), and GM-CSF mRNA (2.3 ± 0.2) when compared to GMF-KO control mice receiving CFA alone. The expression levels of MIP-1 and MIP-2 mRNA remained unchanged. As seen in Fig. 5, both brain and spinal cord of Wt mice at the peak of EAE produced significantly higher levels of iNOS mRNA, 23.4 ± 2.0 -fold and 61.3 ± 6.5 -fold, respectively, when compared with naïve Wt mice (Fig. 5A). GMF-KO mice at the peak of EAE, in contrast, produced lower levels of iNOS mRNA (approximately 3.0-fold) in brain and spinal cord tissue as compared with CFA immunized GMF-KO mice (Fig. 5B). Significantly reduced levels of iNOS mRNA were produced in both brain and spinal cord of GMK-KO mice when compared with Wt mice (Fig. 5C). Overall, these results demonstrate that the mRNA expression of pro inflammatory mediators, IFN- γ , TNF- α , IL-1 β , IL-6, MIP-1, MIP-2, MCP-1, IP-10, GM-CSF and iNOS, was significantly down regulated in the CNS of GMF-KO mice and supported clinical observations of the significantly reduced symptoms of EAE in the KO mice.

3. Discussion

We have reported earlier (Lim et al., 2000; Zaheer and Lim, 1998; Zaheer et al., 2002; Zaheer et al., 2001) that over expression of GMF in astrocytes leads to the secretion of GM-CSF through activation of p38 MAPK and the transcription factor NF-kB. GM-CSF in turn activates microglial cells to produce excess pro inflammatory cytokines, such as TNF- α , IL-1 β , and IL-6 (Zaheer et al., 2006), which consequently destroy oligodendrocytes, the myelin forming cells in the central nervous system. Activated microglia is known to play a crucial role in EAE. Our hypothesis is that GMF is involved in the pathogenesis of EAE. Recently, we tested this hypothesis by inducing EAE in mice with MOG 35–55 peptide and examining the severity of the disease. Our results show a significant decrease in incidence, delay in onset, and reduced severity of EAE in GMF-KO mice compared with Wt mice (Zaheer et al., 2007).

A number of studies employed the transfer of antigen-reactive T cells to analyze the effects in the induction phase in periphery and the effector phase in CNS (Cross et al., 1994; Howard and Miller, 2001; Voskuhl et al., 1996). To better understand the role of GMF in the induction phase versus the effector phase of EAE, we isolated T cells from MOG immunized donor mice and

injected into the non-immunized recipient mice. Our results show that MOG-specific CD4⁺ T cells generated from Wt mice produced significantly less severe EAE in GMF-KO mice as compared to Wt mice. Moreover, MOG-specific CD4⁺ T cells from GMF-KO mice when transferred to Wt mice induced EAE with delayed onset and with significantly less severity. These results confirm our hypothesis that GMF enhances EAE in the effector phase of EAE.

Because EAE is a T cell-mediated disease, we analyzed T cell activation and proliferation. Splenocytes were collected from GMF-KO and Wt mice on day 10 pi, and tested for proliferation by [³H] thymidine incorporation and differentiation into Th1 and Th2 by estimation of specific cytokines (Th1 cytokines such as IFN- γ , TNF- α and Th2 cytokines such as IL-4, IL-10). The inability of T cells from GMF-KO mice to differentiate into effector Th1 strongly supported our hypothesis that GMF is necessary for the induction of EAE. Although MS and EAE are considered to be a CD4⁺ myelin-reactive Th1 cells mediated disease (Steinman, 1996), potential demyelinating antibodies to myelin components do play an important role. Our results show significantly lower levels of circulating anti-MOG antibody in the GMF-KO mice compared to Wt mice.

Expression of high levels of pro inflammatory cytokines and chemokines in the central nervous system are considered to be contributing factors in the initiation and progression of EAE (Godiska et al., 1995; Ransohoff et al., 1996). The activated T cells, microglia and astrocytes produce a variety of pro inflammatory molecules such as TNF- α , IFN- γ , IL-1 β , IL-6, IL-12, IL-17, and IL-23. Cytokines play a critical role in defining the Th1 or Th2 nature of the immune response and regulating inflammation in CNS. Studies in transgenic and knockout mice now provide strong and direct evidence for the involvement of individual cytokine, such as TNF- α , IL-6, IFN- γ (Hamilton et al., 2002; Liu et al., 1998; Samoilova et al., 1998) in EAE. Matejuk et al. (Matejuk et al., 2002) used four cytokines, IL-4, IL-10, IFN- γ and TNF- α , deficient mice to evaluate their role in EAE. They found distinct profiles of cytokines and chemokines in these knockout mice and also a strong association between the expression levels and the overall severity of EAE. Chemokines are potent chemoattractant for activated T cells and regulators of their migration into CNS. Increased levels of several chemokines including RANTES, MIP-1 α and β , TCA3, IP-10, MCP-1 and GM-CSF (Godiska et al., 1995) were found in the spinal cord of EAE. To test the hypothesis that GMF exerts its effects by modulating the cytokine/chemokine profiles in the CNS microenvironment, we examined the expression profiles of pro inflammatory mediators. The mRNA expression levels of cytokines (IFN- γ , TNF- α , IL-1 β , IL-6) and chemokines (MIP-1 β , MIP-2, MCP-1, IP-10, GM-CSF) were significantly reduced in the brain and spinal cord of GMF-KO mice with EAE. These results strongly support our hypothesis that GMF is essential in modulating cytokine/chemokine profiles in the CNS microenvironment.

Excessive amounts of reactive oxygen and nitrogen species (ROS & RNS) are known to produce tissue damage and demyelination (Mitrovic et al., 1994; Smith et al., 1999). Increased CNS levels of ROS and RNS were found in MS and EAE. Recently, it was found (Dasgupta et al., 2002) that iNOS induction by myelin basic protein-primed T cells could produce a significant CNS inflammation. It was also shown that TNF- α and IL-1 β exert neurotoxicity in concert with up regulation of iNOS while in absence of iNOS contributes to neuroprotection and plasticity (Stoll et al., 2000). Amelioration of EAE by inhibitors of iNOS suggests an important role for iNOS-derived NO in the pathogenesis of EAE (Cross et al., 1994; Ding et al., 1998). The role of nitric oxide (NO) is microenvironment and concentration dependent (Beckman and Koppenol, 1996). Excessive NO production from iNOS has been implicated in the cytotoxicity of oligodendrocytes and in the pathology of MS and EAE (Brosnan et al., 1994; Merrill et al., 1993; Sherman et al., 1992). In the present study, our results show enhanced expression of iNOS in Wt mice at the peak of EAE whereas GMF-KO mice produced significantly lower levels of iNOS in both brain and spinal cord.

In summary, we have shown that an absence of endogenous GMF delayed the onset and drastically reduced the severity of EAE induced by MOG 35-55 peptide, as well as in adoptive transfer model. Such a muted incidence of EAE in GMF-KO mice was consistent with significantly reduced expressions of pro-inflammatory cytokines, chemokines and iNOS in brain and spinal cord. Our data strongly supports a crucial role of GMF in the pathogenesis of EAE by modulating the cytokine/chemokine profiles in the CNS microenvironment. Taken together, these results imply that GMF is necessary for the development of EAE and plays a critical role in the induction of the immune responses, as well as in the effector phase of EAE. Our data indicates that GMF enhances the production of Th1-type inflammatory cytokines and that the resistance to EAE development in GMF-KO mice is due to the diminished production of pro inflammatory cytokines and chemokines in the absence of GMF. Our results also provide for the first time, a novel rationale for targeting GMF or the GMF signaling pathway for preventive and therapeutic intervention in MS disease.

4. Experimental Procedures

4.1. Reagents

Myelin oligodendrocyte glycoprotein peptide 35–55 and pertussis toxin were purchased from Sigma-Aldrich, St. Louis, MO. Complete Freund's adjuvant was from Difco, Detroit, MI. CD4 + T cell isolation kit was purchased from Miltenyi Biotech, Auburn, CA. ELISA cytokine antibody pairs "cytose" for mouse IFN- γ (cat# CMC 4033), TNF- α (cat# CMC 3013), IL-4 (cat# CMC 0043) and IL-10 (cat# CMC 0103) were obtained from Invitrogen Corporation, Carlsbad, CA. ThermoScript™ RT-PCR system (Cat #11146-016) for first-strand cDNA synthesis was purchased from Invitrogen Corporation, CA. RNazol B reagent was from Tel-Test, Inc., Friendswood, TX. PCR primers were synthesized at Integrated DNA Technologies (Coralville, IA).

4.2. Mice

GMF-deficient (GMF^{-/-}, GMF knockout) mice were originally produced by homologous recombination with over 80% of the amino acid sequence deleted, as previously described (Lim et al., 2004; Zaheer et al., 2004). GMF- knockout (GMF-KO) mice were maintained by backcross breeding to C57BL/6 for nine generations. These mice were bred and maintained in the animal colony at The University of Iowa. Control wild type (C57BL/6) mice were purchased from Harlan Sprague Dawley, Inc., Indianapolis, IN. The animals were cared for in accordance with the guidelines approved by the IACUC and National Institutes of Health.

4.3. Induction of EAE

For active induction of EAE, mice received 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). Animals injected with complete Freund's adjuvant (CFA) were used as controls. Next day each animal received i.p. injection of 200 ng pertussis toxin. Fifteen C57BL/6.N9, 8–10 week-old, female, GMF knockout (GMF-KO, GMF^{-/-}) mice and an equal number of age and sex matched wild type (GMF^{+/+}) mice were used. The mice were weighed and scored daily in a blinded fashion by two examiners, according to the following scale: score 0, no disease; score 1, loss of weight and 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 (Hilliard et al., 1999). All of 15 Wt mice developed EAE with the mean score of 3.6. In contrast, only 4 of 16 GMF-KO mice showed less severe and delayed onset of EAE as compared to Wt mice. Four GMF-KO mice that developed EAE had a maximum clinical score of only 1.3. Three Wt mice died of EAE whereas no death occurred in GMF-KO group. None of the mice immunized with CSF alone plus pertussis toxin developed the disease (n = 15, data not shown). For passively transferred

EAE, 8 mice in each group were immunized with MOG 35–55 as described above. Ten days later, spleens were harvested, single-cell suspensions were obtained and red blood cells were lysed. The CD4⁺ T cells were isolated using a commercial CD4⁺ T cell isolation kit (Miltenyi Biotech, Auburn, CA) according to the manufacturer's instructions. Stimulation with MOG 35–55 (20 µg/ml) for 72 h was performed. Cells were harvested, washed in PBS, and injected into recipient mice. The incidence rates of EAE in Wt mice and in GMF-KO that received Wt T cells were 100% (8 of 8 mice) and 37.5% (3 of 8 mice), respectively. The EAE incidence rate from transfer of GMF-KO encephalitogenic T cells to either Wt or KO recipient mice was only 25% (2 of 8 mice in each recipient group). The animals were cared for in accordance with the guidelines approved by the IACUC and National Institutes of Health.

4.4. Enzyme-linked immunosorbent assay (ELISA)

The analysis of IFN- γ , TNF- α , IL-4 and IL-10 protein concentration in sera and cell culture supernatants was estimated by sandwich immuno-assay procedure as specified in the manufacturer's protocol (Invitrogen Corporation, Carlsbad, CA). 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 was estimated from a standard curve generated with each run. The lower detection limits of these ELISA are in the range of 8–12 pg/ml. ELISA data are presented as mean values $\pm$ standard deviations and represent more than three independent experiments with similar results.

4.5. T cell proliferation assay

The splenic T cells were isolated from four mice each group (GMF-KO and wild type) on day 10 after immunization with MOG 35–55 as described above. The cells were cultured (2×10^6 cells/ml) in 96-well tissue culture plates in RPMI 1640 medium in the presence of 0–20 µg/ml MOG 35–55. [^3H]Thymidine (0.5 µCi/ml) was pulsed 72 h before harvest. For cytokine assays by ELISA, cells culture supernatants were collected after stimulation with MOG 35–55 (10 µg/ml) for 72 h.

4.6. Anti-MOG 35–55 IgG assay

Microtiter 96-well ELISA plates were coated with MOG 35–55 peptide (5µg/ml, 100µl/well) followed by MOG 35–55 specific IgG detection by ELISA using rabbit anti-mouse IgG as described above.

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

Reverse transcription-polymerase chain reaction (RT-PCR) was carried out as described earlier (Zaheer et al., 1995a; Zaheer et al., 1995b) in detail. For this purpose total RNA was isolated from cultured cells, brain and spinal cord tissue by the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987), using the Trizol reagent. RT-PCR was carried out essentially as described earlier (Zaheer et al., 1995a; Zaheer et al., 1995b). RNA from brain and spinal cord tissue was isolated at the peak of the disease on day 16 and day 26 post immunization (day 16 pi in Wt and day 26 pi in GMF-KO mice) with MOG 35–55 peptide in CFA or with CFA alone. The first strand cDNA synthesis was carried out, using a ThermoScript RT-PCR system kit (GibcoBRL, Life Technologies), in a reaction volume of 20 µl containing 1 µg of total RNA, 100 ng random hexamer, 40 units RNaseOut, and 4 µl 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 (dATP, dCTP, dGTP and dTTP, 1 mM each) 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 × SYBR green PCR master mix (Applied Biosystems) and gene-specific primers in a final volume of 20 µl. The primers were designed with Primer Express software. The following oligonucleotide primers were used: **IFN-γ**, TCAAGTGGCATAGATGTGGAAGAA-3' and 5'-TGGCTCTGCAGGATTTTCATG-3'; **TNF-α**, 5'-CATCTTCTCAAAATTTCGAGTGACAA-3' and 5'-TGGGAGTAGACAAGGTACAACCC-3'; **IL-1β**, 5'-CAACCAACAAGTGATATTCTCCATG-3' and 5'-ATCCACACTCTCCAGCTGCA-3'; **IL-6**, 5'-GAGGATACCACTCCCAACAGACC-3' and 5'-AAGTGCATCATCGTTGTTTCATACA-3'; **IL-4**, 5' ACA GGA GAA GGG ACG CCA T-3' and 5' GAA GCC CTA CAG ACG AGC TCA-3'; **IL-10**, 5' GGT TGC CAA GCC TTA TCG GA -3' and 5' ACC TGC TCC ACT GCC TTG CT -3'; **MIP-1**, 5' CAG CCA GGT GTC ATT TTC CT -3' and 5' AGG CAT TCA GTT CCA GGT CA -3'; **MIP-2**, 5' GAG CTT GAG TGT GAC GCC CCC AGG 3' and 5' GTT AGC CTT GCC TTT GTT CAG TAT C -3'; **MCP-1**, 5' CTT CTG GGC CTG CTG TTC A -3' and 5' CCA GCC TAC TCA TTG GGA TCA -3'; **IP-10**, 5' GCC GTC ATT TTC TGC CTC AT -3' and 5' GCT TCC CTA TGG CCC TCA TT -3'; **iNOS**, 5' CAG CTG GGC TGT ACA AAC CTT -3' and 5' CAT TGG AAG TGA AGC GTT TCG -3'; **GM-CSF**, 5'-GGCCTTGAAGCATGTAGAGG-3' and 5'-GGAGAACTCGTTAGAGACGACTT-3'; **18S**, 5'-TAA GTC CCT GCC CTT TGT ACA CA-3' and 5'-GAT CCG AGG GCC TCA CTA AAC-3'. 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 $x = 2^{-\Delta\Delta C_t}$, where x = fold difference in amount of starting material between two treatment groups (experimental versus control, here trained versus untrained), $\Delta\Delta C_t = \Delta E - \Delta C$, and $\Delta E = C_{t_{exp}} - C_{t_{18Sexp}}$, $\Delta C = C_{t_{control}} - C_{t_{18Scontrol}}$. "Ct" stands for threshold cycle that is the fractional cycle number at which the amount of amplified target reaches a fixed threshold (Livak and Schmittgen, 2001). In some experiments PCR products were electrophoretically analyzed on a 1.8% agarose gel containing ethidium bromide. DNA bands from the gel were excised, purified by GeneClean (BIO 101, La Jolla, Ca) and directly sequenced by the Taq dideoxy terminator cycle sequencing using Applied Biosystems automated DNA sequencing system (model 373A).

4.8. Statistical analysis

Statistical significance was assessed with one-way ANOVA followed by Tukey's procedure using SigmaStat software (SPP, Chicago, IL). A value of $p < 0.05$ was considered statistically significant.

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Fig 1.

(A) Reduced severity, decreased incidence, and delay in onset of EAE in GMF-KO mice. GMF-KO (KO) and wild-type (Wt) mice were immunized with MOG 35–55 peptide in CFA as described in Experimental Procedures. Data are presented as mean EAE score and error bars represent $\pm$ SEM. ($n = 16$ for GMF-KO, and $n = 15$ for Wt mice). These results represent data from three independent experiments. (B) Clinical score of EAE in GMF-KO and Wt recipient mice that received MOG 35–55-reactive T cells generated from KO or Wt mice. Data are presented as the EAE score (mean $\pm$ SEM) in each group of eight recipient mice. Data shown represent two independent experiments.

Fig 2.

(A) T cell proliferation in GMF-KO and Wt mice. CD4⁺ T cells were isolated 10 days after immunization with MOG 35–55 peptide, cultured and re-stimulated with MOG 35–55 peptide *in vitro*. Proliferative response to increasing peptide concentration was determined by [^3H] thymidine incorporation. There was no difference between the T cell proliferative response of GMF-KO and Wt mice. Data shown are means of six wells representing three independent experiments. (B) Cytokine levels in CD4⁺ T cell culture supernatants from MOG 35–55 – immunized Wt and GMF-KO mice. Cells were isolated ten days post immunization and stimulated with MOG 35–55 peptide *in vitro* as described in Experimental Procedures. Cell culture supernatants were collected and the levels of IFN- γ , TNF- α , IL-4 and IL-10 proteins

were determined by ELISA. Data represent the mean $\pm$ S.D. from four independent experiments. *Indicates significant difference between GMF-KO and Wt cells, $p < 0.001$. Differences between the groups were evaluated by ANOVA followed by Tukey's procedure using SigmaStat software. (C) Serum concentration of anti-MOG 35–55 IgG in GMF-KO and Wt mice at the peak of the disease on day 16 and day 26 post immunization (day 16 pi in Wt and day 26 pi in GMF-KO mice) with MOG 35–55 peptide. Serum samples were prepared from peripheral blood of GMF-KO or Wt mice and MOG 35–55 antibody was determined by ELISA. The results are expressed as OD_{450 nm} values. Significantly lower levels of anti-MOG 35–55 IgG were detected in GMF-KO mice compared to Wt mice at day 16 and 26. *Indicates significant difference (ANOVA) between the results of GMF-KO and Wt mice.

Fig 3.

Reduced levels of circulating cytokines, IFN- γ and TNF- α , in GMF-KO mice immunized with MOG 35–55. Serum concentration of IFN- γ , TNF- α , IL-4 and IL-10 protein were determined by ELISA in GMF-KO and Wt mice at the peak of the disease on day 16 and day 26 post immunization (day 16 pi in Wt and day 26 pi in GMF-KO mice) with MOG 35–55. Pro inflammatory cytokines, IFN- γ and TNF- α , levels at day 16 pi were significantly lower in GMF-KO mice compared to Wt mice, *p < 0.001. No significant differences were found in IL-4 and IL-10 levels between GMF-KO and Wt mice. Assays had a minimum sensitivity of 10–20 pg/ml. Data represent the mean $\pm$ S.D. from three independent experiments.

Fig 4.

Quantitative real-time RT-PCR analysis of cytokine and chemokine mRNA in brain of Wt and GMF-KO mice. The mRNA expression levels were determined by quantitative real-time RT-PCR in total RNA isolated from brains of Wt mice and GMF-KO mice. Cytokine/chemokine mRNA levels were estimated by determining ΔC_T , the difference between the C_T of the cytokine of interest and the C_T of the endogenous 18S rRNA control gene. (A) The expression of cytokine mRNA in Wt brain. (B) The mRNA expression levels of pro inflammatory chemokines, MIP-2, MCP-1, IP-10, and GM-CSF in Wt mice brain. (C) The expression of cytokine mRNA in GMF-KO brain. (D) The expression of chemokine mRNA in GMF-KO brain. (E) Cytokine mRNA expression in spinal cord of Wt mice. (F) Chemokine mRNA expression in spinal cord of Wt mice. (G) Cytokine mRNA expression in spinal cord of GMF-KO mice. (H) Chemokine mRNA expression in spinal cord of GMF-KO mice. Data shown are the mean $\pm$ S.D. of three replications with and statistical significance of the differences from the controls (CFA-immunized) are marked (* = $p < 0.05$, ANOVA).

Fig 5.

Relative iNOS mRNA levels in brain and spinal cord of Wt and GMF-KO mice. Quantitative real-time RT-PCR analysis of iNOS mRNA was carried out as described in Fig. 4. **(A)** Wt mice. **(B)** GMF-KO mice. **(C)** Relative iNOS mRNA levels in brain and spinal cord of Wt and GMF-KO mice at the peak of the disease on day 16 and day 26-post immunization (day 16 pi in Wt and day 26 pi in GMF-KO mice) with MOG 35-55 peptide. Data represent the mean $\pm$ S.D. from three independent experiments and statistical significance of the differences from the controls are marked (* = $p < 0.001$, ANOVA).