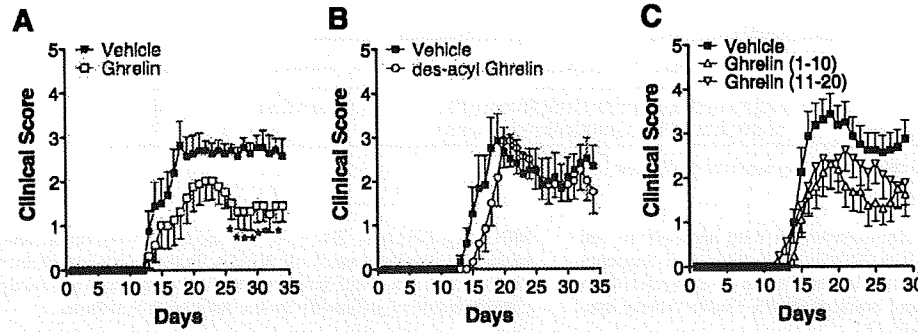

FIGURE 1. Effect of ghrelin on actively induced EAE. EAE was induced in female B6 mice (n = 8 in each group of the three experiments) by immunization with MOG_{35-55}. *A*, The mice were treated every other day starting at the day of immunization with 5 μg/kg ghrelin, while controls were administrated with the vehicle, 0.9% saline, alone. *B*, The mice were injected from day 1 every other day with 5 μg/kg des-acyl ghrelin, whereas controls were subjected to 0.9% saline injections. *C*, Following an alternative protocol, mice were treated from days 1–10 (induction phase treatment) or from days 11–20 (effector phase treatment) with 5 μg/kg ghrelin and in-between with 0.9% saline, while controls were treated every day with 0.9% saline injections. Data represent mean ± SEM. *, Significant differences between the groups ($p < 0.05$; Mann-Whitney U test).

FACSAria (BD Biosciences). The total RNA was extracted from the isolated cells and was subjected to reverse transcription and real-time PCR.

In vitro effect of ghrelin on microglia cells treated with LPS

Mononuclear cells were prepared from brains of untreated non-EAE mice incubated with Liberase Blendzyme 3 and DNase I as described above and were isolated on 40%–80% discontinuous Percoll gradients. Isolated cells were suspended in DMEM supplemented with 10% FCS and cultured in 96-well flat bottom plates at 2×10^5/well in the presence of ghrelin (10^{-6} M) overnight and later stimulated with LPS at different doses (0.01, 0.1 μg/ml). After 5 h of incubation at 37°C, supernatants were collected and the levels of TNF-α were detected by using a sandwich ELISA.

Statistical analysis

The differences in the clinical score between ghrelin-, des-acyl ghrelin-, and sham-treated groups were analyzed by the nonparametric Mann-Whitney U test. FACS analysis, real-time PCR, ELISA, and proliferation data were subjected to two-way ANOVA. In case of significant differences, a Fisher post hoc test was applied. Probability values of <0.05 were considered as statistically significant.

Results

Ghrelin inhibits EAE

To explore the modulatory effects of ghrelin on inflammatory demyelinating diseases, we employed a model of EAE actively induced in B6 mice with MOG_{35-55}. Although classical forms of EAE are typically characterized by acute paralysis followed by complete recovery, this EAE model shows persistent paralysis with partial recovery as a reflection of persistent inflammatory demyelination in the CNS (21, 22). In the first series of experiments, we injected 0.5, 5, or 50 μg/kg ghrelin to the mice every other day from day 1 to 35 postimmunization, while the control mice were injected with 0.9% saline. The results showed that the continuous injections of 5 μg/kg ghrelin suppressed most efficiently the clinical signs of EAE (Fig. 1*A*), whereas a lower (0.5 μg/kg) or a higher dose (50 μg/kg) showed only a marginal effect (data not shown). The treatment with 5 μg/kg ghrelin did not significantly alter either the onset or peak score of EAE. However, significant differences were noted in mean clinical score after day 25 postimmunization between the ghrelin-treated and the control mice (Fig. 1*A*).

Moreover, the effect of ghrelin on EAE was specific as des-acyl ghrelin, an acyl-modified ghrelin, which lacks the n-octanoic acid on the third serine, and consequently its binding ability to GHS-R (7) (Table I) had no modulatory effect on EAE at any concentration examined (Fig. 1*B* and Table II). Thus, the discrepant results obtained with ghrelin and des-acyl ghrelin indicate that ghrelin treatment would ameliorate the clinical course of EAE via activation of the GHS-R.

To further characterize the effects of ghrelin on EAE, we next examined if treatment lasting for a shorter duration may also be immunomodulatory in vivo. We injected 5 μg/kg ghrelin every day from day 1 to 10 postimmunization (roughly corresponding to the induction phase) or from day 11 to 20 (roughly corresponding to the effector phase). As shown in Fig. 1*C*, both protocols showed similar levels of disease suppression, although it was less notable than the continuous treatment from day 1 to 35 (Table II).

Ghrelin does not influence cellular infiltration into CNS

In the previous results on prophylactic or therapeutic treatment of EAE, clinical suppression of EAE was generally associated with a significant reduction of cellular infiltration in the CNS (23). To clarify if histological manifestation of EAE is also suppressed by ghrelin treatment, we treated MOG_{35-55}-immunized B6 mice with 5 μg/kg ghrelin or 0.9% saline every other day and prepared sections of spinal cords at the peak of disease (day 17 after immunization) (Fig. 2). Clinical signs were milder in the ghrelin-treated mice compared with saline-treated ones. However, histology of the spinal cord sections with H&E staining revealed equivalent levels of cellular infiltration in ghrelin- and saline-treated mice. To confirm this, we isolated mononuclear cells from spinal cords of the

Table II. *Clinical scores of EAE treated with ghrelin or des-acyl ghrelin following different treatment protocols*[a]

Treatment	Incidence	Mean Day of Onset ± SEM	Mean Maximal Score ± SEM	Mean Cumulative Score ± SEM
Vehicle[b]	8/8	16.38 ± 1.13	3.75 ± 0.33	55.44 ± 7.14
Ghrelin[b]	7/8	17.86 ± 1.30	3.29 ± 0.33	36.71 ± 9.99
Vehicle[b]	6/8	18.83 ± 2.55	3.67 ± 0.40	49.33 ± 12.99
Des-acyl ghrelin[b]	6/8	18.00 ± 0.71	3.80 ± 0.44	49.05 ± 8.09
Vehicle[c]	7/8	15.14 ± 0.51	4.43 ± 0.07	50.43 ± 3.10
Ghrelin (1–10)[c]	6/8	16.00 ± 0.73	3.17 ± 0.53	34.00 ± 7.25
Ghrelin (11–20)[c]	7/8	16.29 ± 1.25	3.50 ± 0.45	38.72 ± 8.79

[a] The table shows the results of three separate experiments (n = 8 mice in each group of the three experiments).

[b] After induction of EAE with MOG_{35-55}, mice were treated in two different experiments following the standard protocol of every other day s.c. treatment with 5 μg/kg ghrelin or 5 μg/kg des-acyl ghrelin. The controls were injected with 0.9% saline (vehicle).

[c] Following an alternative protocol, we treated the mice from days 1–10 (induction phase treatment) or from days 11–20 (effector phase treatment) with 5 μg/kg ghrelin and in-between with 0.9 % saline, while controls were injected every day with 0.9% saline only. Data represent mean ± SEM.

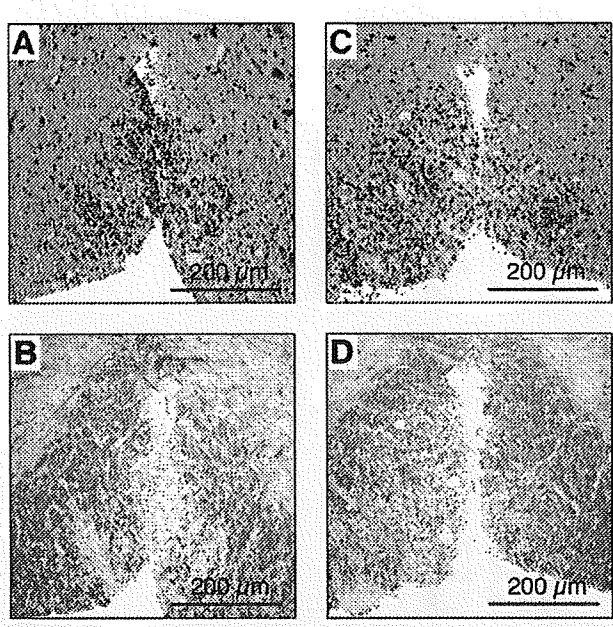

FIGURE 2. Histopathological assessment of the spinal cord of EAE mice. Spinal cords from EAE mice (n = 5/group) were removed on day 17 postimmunization as described in *Material and Methods*. The spinal cord sections from sham- (*A* and *B*) and ghrelin-treated (*C* and *D*) mice were stained in with H&E in the upper panels or Luxol fast blue in the lower ones. Representative sections are shown.

mice at the peak of disease and enumerated the number of the lymphoid cells. Notably, the total cell number was slightly elevated in the ghrelin-treated mice (1.40×10^6/mouse) compared with the saline-treated mice (1.05×10^6/mouse). To further analyze the effects of ghrelin on the formation of CNS inflammation, we evaluated the cellular composition of the CNS-derived lymphocytes by using FACS. Although there was a trend that $CD4^+$ and $CD8^+$ T cell numbers are increased in the lesions of ghrelin-treated mice as compared with saline-treated mice (Fig. 3*A*), it did not reach the level of statistic significance. It was also noted that ghrelin treatment did not alter the number of NK cells

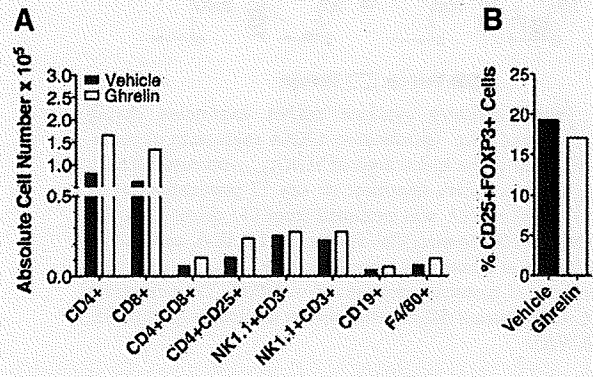

FIGURE 3. Quantification of spinal cord cellular infiltrates by flow cytometry. *A*, The cells were isolated from spinal cords of ghrelin- and sham-treated mice on day 17 postimmunization and subjected to flow cytometer analysis as described in *Materials and Methods*. Data are representative of two independent experiments and presented as absolute cell number (n = 8 mice/group in each experiment). *B*, The proportion of $CD25^+FOXP3^+$ cells in the $CD4^+$ T cell population isolated from spinal cord mononuclear cells was analyzed by flow cytometry 20 days after immunization. Data represent two independent experiments (n = 5).

Table III. *Cytokine production and proliferation of MOG_{35-55}-specific T cells after ghrelin treatment*[a]

Treatment	CPM ± SEM	Cytokine Production (pg/ml) INF-γ ± SEM	IL-17 ± SEM	IL-4 ± SEM
Vehicle	47,590 ± 10,988	2,087 ± 487	820 ± 211	ND
Ghrelin	36,663 ± 9,058	2,883 ± 615	674 ± 148	ND

[a] Mice were immunized with MOG_{35-55} and treated with 5 μg/kg ghrelin or 0.9% saline everyday from day 1 to 10 (n = 3/group). Popliteal and inguinal LN cells were harvested on day 11 after immunization and stimulated with 10 μg/ml MOG_{35-55}. CPM marks the proliferative response to MOG_{35-55}. The cytokines were measured in the supernatant by sandwich ELISA after 72 h of stimulation. Data represent mean ± SEM of duplicate samples from one out of three independent experiments. ND, Not detectable.

($NK1.1^+CD3^-$), NKT cells ($NK1.1^+CD3^+$), B cells ($CD19^+$), or macrophages ($F4/80^+$) in the spinal cord lesions. The proportions of $CD25^+FOXP3^+$ cells in the $CD4^+$ T cell population isolated from spinal cords were not altered in ghrelin-treated mice (Fig. 3*B*). In parallel, we also examined the composition of lymphoid cells obtained from spleen, LN, and thymus. Again, we could not reveal any significant change in the subsets of lymphocytes in ghrelin-treated mice (data not shown). Concordant with the histological findings, these data imply that ghrelin did not ameliorate clinical EAE by reducing the numbers of inflammatory cells in the CNS, but rather by regulating the inflammatory potential of the CNS infiltrates.

Ghrelin does not inhibit the induction of MOG_{35-55}-reactive T cells

To elucidate the immunomodulatory mechanism of ghrelin, we examined the cytokine production and proliferative response of draining LN cells to MOG_{35-55} that were obtained from MOG_{35-55}-sensitized mice treated for 10 days every day with ghrelin or saline. The LN cells were collected on day 11 after immunization and stimulated with MOG_{35-55} in vitro. Accordingly, we harvested the supernatant and measured the levels of IFN-γ, IL-17, and IL-4 by using ELISA. Although the IL-4 concentration was under the detection level, IFN-γ and IL-17 could be detected in the MOG_{35-55}-stimulated culture supernatant (Table III). There was no significant difference in the level of IFN-γ and IL-17 when we compared ghrelin-treated and saline-treated groups. Furthermore, ghrelin-treated mice did not differ from saline-treated mice in the proliferative response of the draining LN cells to MOG_{35-55}. We also examined the frequency of $CD4^+CD25^+$ $FOXP3^+$ regulatory T cells in the lymph nodes and spleens using flow cytometry and did not find significant differences between ghrelin-treated and saline-treated mice (data not shown). These results indicate that in vivo ghrelin treatment did not inhibit the induction of MOG_{35-55}-reactive T cells.

Ghrelin does not affect induction of pathogenic autoimmune T cells

To further confirm that MOG_{35-55}-reactive T cells are normally induced in ghrelin-treated mice, we evaluated if the ability of the MOG_{35-55}-sensitized lymphoid cells, obtained from MOG_{35-55}-immunized mice, to transfer EAE into naive mice could be affected by in vivo ghrelin treatment. To this aim, we immunized donor mice with MOG_{35-55} and treated them every day with ghrelin or saline from immunization up to day 10. Next day, we pooled lymphocytes from spleen and LN and cultured them in the presence of MOG_{35-55}. Three days later, $CD4^+$ T cells were purified and injected into recipient mice as described in *Materials and Methods*. It was theoretically possible that in vivo ghrelin treatment does not

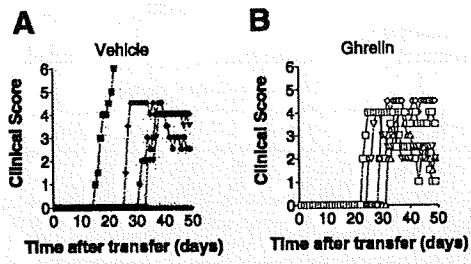

FIGURE 4. Effects of ghrelin treatment on the induction of encephalitogenic T cells. MOG_{35-55}-sensitized lymphoid cells were derived from MOG_{35-55}-immunized and (*A*) saline- or (*B*) ghrelin-treated mice (n = 15/group). The cells were stimulated with MOG_{35-55} and $CD4^+$ T cells were separated 3 days later for passive transfer of EAE into naive mice (n = 5/group). Data represent individual EAE score for each mouse.

inhibit induction of MOG_{35-55}-reactive T cells, but would prohibit the ability to cause EAE in vivo. In postulating that this could happen, $CD4^+$ T cells from ghrelin-treated donors should be less encephalitogenic than those from saline-treated mice. The results showed that transfer of activated $CD4^+$ T cells either derived from saline- or ghrelin-treated donors induced passive EAE in the recipients, showing approximately the same clinical course and severity (Fig. 4). Thus, it can be concluded that ghrelin treatment does not affect the induction of encephalitogenic MOG_{35-55}-reactive $CD4^+$ T cells.

Ghrelin decreases mRNA levels of proinflammatory cytokines in the CNS

After demonstrating that ghrelin does not suppress the infiltration of inflammatory cells in the spinal cord, we wondered whether the cytokine milieu in the ghrelin-treated mice could be significantly altered. To answer the question, we analyzed the mRNA levels of pro- and antiinflammatory cytokines (IFN-γ, TNF-α, IL-1β, IL-6, IL-4, IL-10, and TGF-β) in the spinal cord, spleen, LN, and thymus of ghrelin- and saline-treated mice at the peak of disease (day 17) by using quantitative PCR. Although ghrelin treatment had no effect on the mRNA levels of IL-4, IL-10, and IFN-γ in the spinal cord, spleen, LN, and thymus (data not shown), we found significantly reduced levels of TNF-α ($p < 0.0015$), IL-1β ($p < 0.025$), and IL-6 ($p < 0.025$) in the spinal cord of ghrelin-treated mice, compared with saline-treated ones (Fig. 5*A*). In contrast, the level of TGF-β showed a trend for slight elevation in the spinal cord. We also found a diminished level of TNF-α mRNA ($p < 0.0001$) in the spleen of ghrelin-treated mice (Fig. 5*B*), whereas we saw no significant change in any of the cytokines that we measured in LN or thymus of ghrelin-treated mice (Fig. 5, *C* and *D*). Because TNF-α, IL-1β, and IL-6 mRNAs were selectively down-regulated in the spinal cord, we suspected that monocytes could be potential target cells in the ghrelin-mediated EAE suppression. This idea was consistent with the fact that ghrelin treatment did not inhibit the induction of MOG_{35-55}-reactive T cells.

Ghrelin suppresses the proinflammatory cytokine production of LPS-stimulated monocytes

To verify the postulate that in vivo treatment with ghrelin may ameliorate EAE by targeting monocytes, we examined in vitro effects of ghrelin on the monocytic cell line RAW 264.7 that robustly produce proinflammatory cytokines when stimulated with LPS. The RAW 264.7 line cells were first exposed to various doses of ghrelin for 1 h and then stimulated with LPS. We harvested the supernatant 2 h later and measured the levels of TNF-α and IL-6 by ELISA. The results revealed that prior exposure to ghrelin

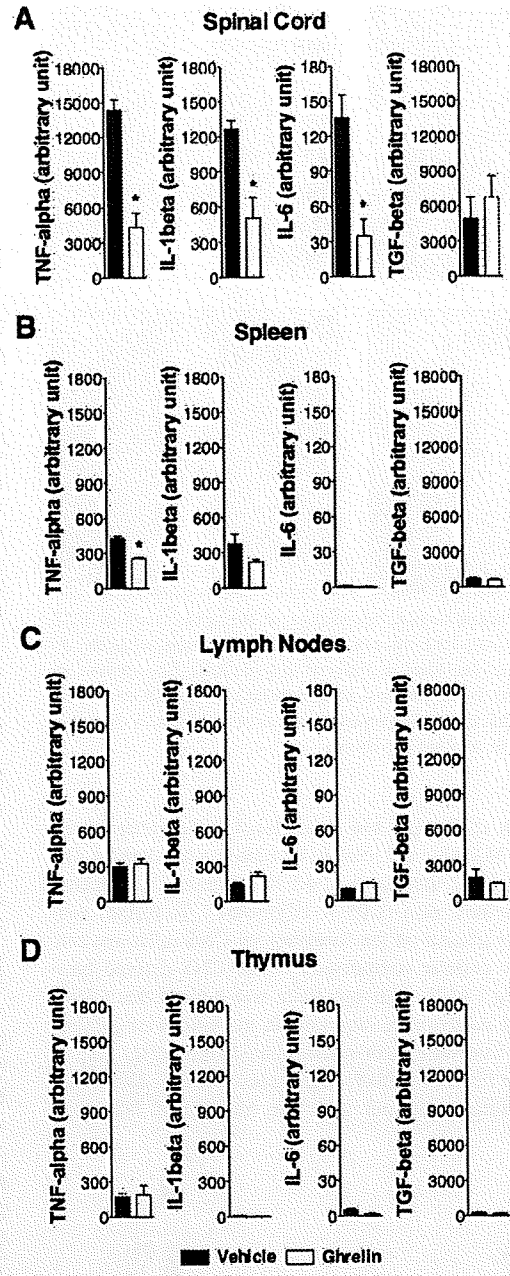

FIGURE 5. Proinflammatory cytokine mRNA expression during EAE in ghrelin-treated mice. Quantitative mRNA expression of proinflammatory cytokines in the spinal cord of MOG_{35-55}-immunized mice subjected to ghrelin or saline treatment on day 17 postimmunization (n = 5/group). Total mRNA was extracted from (*A*) spinal cord, (*B*) spleen, (*C*) LN, and (*D*) thymus. The TNF-α, IL-1β, IL-6, and TGF-β mRNA expression was measured by real-time PCR. Data are presented as relative amount of transcript normalized to HPRT. Data represent mean ± SEM. *, Significant differences between the groups ($p < 0.025$; two-way ANOVA).

would significantly suppress the production of TNF-α ($p < 0.02$) and IL-6 ($p < 0.05$) by LPS-stimulated RAW 264.7 cells in a dose-dependent manner (Fig. 6). The inhibitory effect of ghrelin was very potent, as in addition to the effects on LPS-stimulated monocytes, even the basal production of TNF-α ($p < 0.008$) and IL-6 ($p < 0.03$) was significantly reduced by in vitro ghrelin treatment. Given that in vivo treatment with ghrelin could suppress the

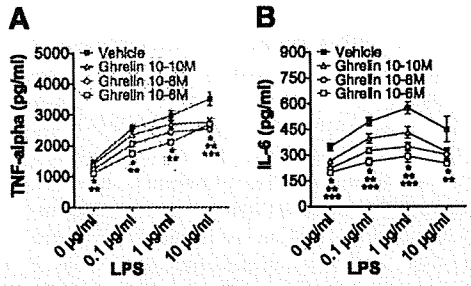

FIGURE 6. Effect of ghrelin on the proinflammatory cytokine production of LPS-stimulated monocytes. The monocytes were treated with various concentrations of ghrelin (10^{-6} M, 10^{-8} M, 10^{-10} M) 1 h before stimulation with 0.1, 1.0, and 10 μg/ml LPS. The (*A*) TNF-α and (*B*) IL-6 production was measured 2 h after LPS stimulation by sandwich ELISA. Data represent mean ± SEM of duplicate samples from one out of three independent experiments. Significant differences at 10^{-6}, 10^{-8}, and 10^{-10} M ($p < 0.05$; two-way ANOVA) are depicted as *, **, and ***, respectively.

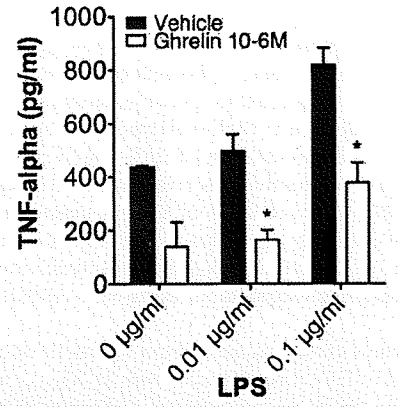

FIGURE 8. Effect of ghrelin on the proinflammatory cytokine production of LPS-stimulated microglia. The microglia cells were treated with ghrelin (10^{-6} M) overnight and later stimulated with 0.01 and 0.1 μg/ml LPS. Five hours after stimulation, the TNF-α production was measured using ELISA. Data represent mean ± SEM of duplicate samples from one out of two independent experiments. *, Significant differences between the groups ($p < 0.05$; two-way ANOVA).

development of EAE without altering histological EAE or T cell-derived cytokine balance, the ghrelin-mediated suppression of monocyte-produced TNF-α and IL-6 would strongly support the postulate that monocytes are the main target cells in ghrelin-mediated suppression of EAE.

Ghrelin inhibits the expression of proinflammatory cytokines in microglia

The proinflammatory cytokines are known to be produced not only by CNS-infiltrating macrophages but also by T cells and microglia in the course of EAE. To investigate which cells are important in the ghrelin-mediated suppression of EAE, we first examined the expression of proinflammatory cytokines in macrophages. Unexpectedly, the mRNA of IL-1β, IL-6, and TNF-α did not alter in CNS-infiltrating macrophages of ghrelin-treated mice compared with the control mice (Fig. 7*A*). We next examined the expression of these cytokines in other cell types also known as a source of inflammatory cytokines and found reduced expression of these cytokines in microglia (Fig. 7*B*). Additionally, the expression of inflammatory cytokines was decreased in CNS-infiltrating T cells (Fig. 7*C*). Hence, these results suggest that microglia might play a crucial role in ghrelin-mediated inhibition of EAE.

Ghrelin inhibits the proinflammatory cytokine production of LPS-stimulated microglia

We next examined the effect of ghrelin on microglia. To test whether ghrelin directly affects microglia, we isolated mononuclear cells from the brains of untreated mice. In untreated non-EAE mice, most (~77%) of the brain mononuclear cells were CD11b$^+$ cells, and the majority of CD11b$^+$ cells (~95%) were considered as CD45^{low} microglia cells. Among these mononuclear cells, CD19$^+$ B cells were <0.1% and CD3$^+$CD45$^+$ T cells were 1–1.5%. We cultured the isolated mononuclear cells in the presence of ghrelin overnight and stimulated them with LPS in different doses for 5 h. The TNF-α levels in the culture supernatant were measured by using ELISA. In the presence of ghrelin, the TNF-α levels were significantly reduced (Fig. 8). These results suggest that ghrelin directly affects microglia by reducing the production of inflammatory cytokines.

Discussion

Starvation is known to have immunosuppressive effects (24–26). Although little was known about the mechanistic link between starvation and immunity, recent studies have shed light on the immunomodulatory potency of a range of feeding regulatory hormones such as leptin and NPY. For example, serum leptin is decreased after acute starvation in parallel with immunosuppression or Th2 bias, whereas exogenous leptin would correct the altered Th1/Th2 balance toward Th1 (27, 28). In contrast, NPY is increased after starvation. Exogenous NPY would shift the Th1/Th2 balance toward Th2 and can ameliorate the severity of EAE (29). Interestingly, both peptide hormones are linked to ghrelin in an endocrine feedback system (30). Ghrelin itself is increased after

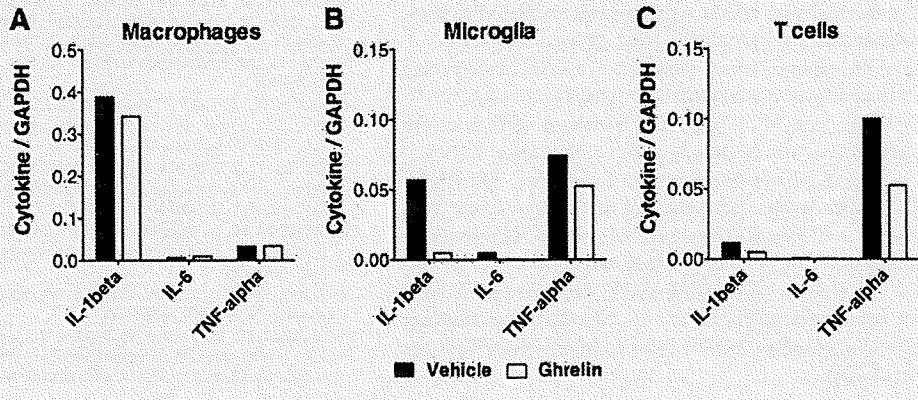

FIGURE 7. Effect of ghrelin on proinflammatory cytokine mRNA expression in infiltrating cells and microglia. Total mRNA was extracted from (*A*) macrophages, (*B*) microglia, and (*C*) T cells obtained on day 20 postimmunization from the spinal cords of MOG_{35-55}-immunized mice treated with ghrelin or saline. The IL-1β, IL-6, and TNF-α mRNA expression levels were measured by real-time PCR. Data are presented as relative amount of transcript normalized to the housekeeping gene GAPDH.

starvation, and it can potently stimulate the release of NPY in the CNS (12). Moreover, ghrelin shows antagonistic effects against leptin (31). Although the available data on the action of ghrelin on leptin or NPY may not be extrapolated to speculate about its role in the immune system, we decided to explore whether ghrelin may exhibit beneficial effects in the modulation of EAE. Furthermore, ghrelin was reported to have protective effects on endotoxic shock in rats (32). Additionally, the wide range of GHS-R expression within the immune cells strongly suggested the immunomodulatory potential of ghrelin (6). Considering its endocrine interactions, ghrelin becomes an interesting candidate for the in vivo modulation of EAE.

To evaluate the effects of ghrelin on the immune system in vivo, we used the representative EAE model induced with MOG_{35-55} in B6 mice. Subcutaneous injections of ghrelin significantly suppressed EAE severity, especially after the peak of disease, while the EAE onset occurred almost similarly in both ghrelin- and sham-treated mice. Priming phase treatment (days 1–10) as well as effector phase treatment (days 11–20) also showed disease-suppressing effects, suggesting a modulatory role of ghrelin during all phases of disease. The unacylated ghrelin form, des-acyl ghrelin, failed to suppress EAE, demonstrating that the disease suppression was mediated by the GHS-R.

The histological findings at day 17 were similar in all animals regardless of the applied treatment. The inflammatory cell infiltration and demyelination occurred in both groups, suggesting a ghrelin effect independent of cell trafficking at the peak of disease. Moreover, we found by FACS analysis that the number of mononuclear cells isolated from the spinal cord and their composition did not significantly alter among ghrelin- and sham-treated mice at the same time point. Our data showed no statistically significant changes in the examined cell subsets, which supported the histological findings of unaffected immune cell traffic to the CNS. This discrepancy between analogous inflammatory status in the spinal cord on the one hand and less severe disease on the other hand in ghrelin-treated mice was remarkable, suggesting cytokine regulation as the possible mechanism of EAE suppression.

Leptin and NPY both influence the Th1/Th2 balance in opposing directions (27–29). Since ghrelin is the most potent NPY-releasing hormone and NPY suppresses EAE by a Th2 bias (29), we examined whether ghrelin affects the Th1/Th2 balance similar to NPY and if its potential mechanism of EAE suppression is primarily mediated on immune cells or secondarily through NPY release. To investigate the effect of ghrelin on the cytokine balance, we measured the cytokine responses of MOG_{35-55}-primed T cells from mice treated with ghrelin or saline. The evaluated IFN-γ, IL-17, and IL-4 levels as well as the proliferative response did not significantly alter between ghrelin- and sham-treated mice. Underlying these observations, we conclude that the suppression of EAE mediated by ghrelin does not affect the T cell-derived cytokine balance. To further address whether ghrelin acts via the NPY pathway, we determined the encephalitogenic potential of $CD4^+$ T cells from ghrelin-treated mice to cause passive EAE in syngeneic recipients. We treated donor animals with ghrelin or saline for 10 days after priming with MOG_{35-55}, and lymphoid cells from the mice were stimulated with MOG_{35-55}. Three days later, $CD4^+$ T cell blasts were isolated and transferred to naive mice. The $CD4^+$ T cells from ghrelin-treated mice did not differ from those from saline-treated mice in the ability to mediate passive EAE, indicating that ghrelin does not primarily affect induction of encephalitogenic $CD4^+$ T cells in vivo. While NPY attenuates EAE by a Th2 bias of encephalitogenic $CD4^+$ T cells (29), our findings likely suggest that ghrelin interacts independently of NPY in the amelioration of EAE.

To further clarify the mechanism of ghrelin-mediated EAE suppression, we examined the mRNA levels of several cytokines of ghrelin- and sham-treated mice at the peak of disease. Our data demonstrate significantly reduced levels of the proinflammatory cytokines TNF-α, IL-1β, and IL-6 in the spinal cord and lower levels of TNF-α in the spleen of ghrelin-treated mice. In contrast, the level of TGF-β showed a trend for slight elevation in the spinal cord. The importance of TNF-α for initiating and sustaining inflammation is well described, as well as its essential role in the development of acute EAE (33, 34). The proinflammatory role of IL-1β and IL-6 in the immunopathology of EAE is also generally accepted (35–38). Thus, the inhibition of TNF-α, IL-1β, and IL-6 must be considered as an important mechanism in the ghrelin-mediated EAE suppression.

Given the selective down-modulation of the proinflammatory cytokines, we suspected that monocytes could be potential target cells in the ghrelin-mediated EAE suppression. However, the analysis of infiltrating cells and residential microglia revealed that the suppression of proinflammatory cytokines was prominently led by microglia. A decreased expression of these cytokines was also observed in infiltrating T cells. Considering that the transfer of T cells obtained from ghrelin-treated mice induced a similar disease course compared with control mice, the reduction of proinflammatory cytokines in microglia might be important in the ghrelin-mediated suppression of EAE.

In conclusion, the present study demonstrates for the first time to our knowledge that the gastric hormone ghrelin suppresses actively induced EAE by inhibiting production of the proinflammatory cytokines TNF-α, IL-1β, and IL-6 with microglia as the main target cells. These findings support an antiinflammatory property of ghrelin, shedding light on its role in immune-endocrine interactions. Consequently, we speculate that ghrelin may serve as an antiinflammatory drug to control human CNS pathology involving the production of proinflammatory cytokines.

Disclosures

The authors have no financial conflicts of interest.

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Immunopathology and Infectious Diseases

Carcinoembryonic Antigen-Related Cell Adhesion Molecule 1 Modulates Experimental Autoimmune Encephalomyelitis via an iNKT Cell-Dependent Mechanism

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Carcinoembryonic antigen-related cellular adhesion molecule 1 (CEACAM1) is a CEA family member that has been reported to have an important role in the regulation of Th1-mediated colitis. In this study, we examined the role of CEACAM1 in an animal model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE). Treatment of C57BL/6J mice with CEACAM1-Fc fusion protein, a homophilic ligand of CEACAM1, inhibited the severity of EAE and reduced myelin oligodendrocyte glycoprotein-derived peptide (MOG_{35-55})-reactive interferon-γ and interleukin-17 production. In contrast, treatment of these animals with AgB10, an anti-mouse CEACAM1 blocking monoclonal antibody, generated increased severity of EAE in association with increased MOG_{35-55}-specific induction of both interferon-γ and interleukin-17. These results indicated that the signal elicited through CEACAM1 ameliorated EAE disease severity. Furthermore, we found that there was both a rapid and enhanced expression of CEACAM1 on invariant natural killer T cells after activation. The effect of CEACAM1-Fc fusion protein and anti-CEACAM1 mAb on both EAE and MOG_{35-55}-reactive cytokine responses were abolished in invariant natural killer T cell–deficient $J\alpha18^{-/-}$ mice. Taken together, the ligation of CEACAM1 negatively regulates the severity of EAE by reducing MOG_{35-55}-specific induction of both interferon-γ and interleukin-17 via invariant natural killer T cell-dependent mechanisms. *(Am J Pathol 2009, 175:1116–1123; DOI: 10.2353/ajpath.2009.090265)*

Carcinoembryonic antigen-related cellular adhesion molecule 1 (CEACAM1), also known as CD66a, is one of the carcinoembryonic antigen family members and is expressed in epithelial cells, endothelial cells, and hematopoietic cells such as monocytes, dendritic cells, natural killer (NK) cells, B cells, and activated T cells.[1–4] It is involved in intercellular adhesion through homophilic or heterophilic interactions and mediates regulatory functions in cellular growth and differentiation. Several splice variants of CEACAM1 have been detected, that differ with respect to the number of extracellular immunoglobulin-like domains, membrane anchorage, and the length of their cytoplasmic tail.[3] Isoforms of CEACAM1 with a long cytoplasmic tail (CEACAM1-L) contain two immunoreceptor tyrosine-based inhibitory motifs and have been shown to negatively regulate epithelial cell activation and tumor cell growth.[3–5] Recently, the specific function of CEACAM1 as a regulator of T cells has been reported *in vitro* and *in vivo*.[6–12] Mice treated with CEACAM1-Fc fusion protein, a homophilic ligand for CEACAM1 that stimulates the signal from CEACAM1, exhibited an immunosuppressive effect on Th1-mediated colitis *in vivo*, with reduced interferon (IFN)-γ production and T-bet activation.[12] However, the significance of CEACAM1 on other inflammatory autoimmune disease models remains unclear.

Experimental autoimmune encephalomyelitis (EAE) is an inflammatory demyelinating disease induced by sensitization against central nervous system (CNS) components such as myelin oligodendrocyte glycoprotein

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(MOG).[13] Because the neurological signs of paralysis can be monitored continuously, and demyelinating lesions resemble those found in multiple sclerosis, EAE is considered an animal model of the human demyelinating disease multiple sclerosis.[13–16] Numerous studies have reported that EAE is mediated by $CD4^+$ Th1 cells that produce IFN-γ.[13–16] Recently, this idea was questioned because animals deficient in IFN-γ, IFN-γ receptor, or signal transducer and activator of transcription 1 were still found to develop EAE.[17–21] These data led the identification of an interleukin (IL)-23 derived population of Th cells, IL-17-producing Th17 cells, as alternative potent inducers of severe autoimmunity, including EAE.[22–24] However, mice deficient in T-bet and signal transducer and activator of transcription 4, which thus lack Th1 cells, but have large numbers of Th17 cells, are still resistant to EAE.[21,25] Additionally, Th1 and Th17 cells are observed in the CNS at the peak of EAE and diminish after the recovery.[26] It has now been described that Th1 and Th17 cells might cooperate to induce the development of EAE.[27–29] Thus, elucidation of the mechanisms that regulate the production of both Th1 and Th17 cytokines is important in relation to the regulation of EAE.

In this study, we investigated the role of CEACAM1 in EAE either by CEACAM1 ligation with a homophilic ligand for CEACAM1 (CEACAM1-Fc fusion protein), or by blocking with a CEACAM1-specific antibody, AgB10. Here, we demonstrate that signaling through CEACAM1 suppressed MOG-derived peptide (MOG_{35-55})-induced EAE associated with a reduction in MOG_{35-55}-specific T cell production of IFN-γ and IL-17. Moreover, we have identified invariant natural killer T (iNKT) cells as a critical component in CEACAM1-mediated suppression of EAE. iNKT cells are an unique subset of CD1-restricted T cells that express an invariant T cell receptor (TCR) α chain, composed of Vα14-Jβ18 segments in mice and Vα14-Jβ18 segments in humans, and use a restricted set of Vβ genes.[30–31] Due to the ability to produce a wide variety of cytokines, iNKT cells are thought to play regulatory roles in autoimmune diseases.[32] CEACAM1-mediated suppression of EAE was not observed in iNKT cell-deficient $J\alpha18^{-/-}$ mice, and MOG_{35-55}-specific T cell production of IFN-γ and IL-17 was not modified in $J\alpha18^{-/-}$ mice when treated with either CECAM1-Fc fusion protein or AgB10.

Materials and Methods

Animals and Reagents

C57BL/6J (B6) mice were obtained from CLEA Japan Inc. (Tokyo, Japan). $J\alpha18^{-/-}$ mice were kindly provided by Dr. M. Taniguchi (RIKEN, Tokyo, Japan). All animals were maintained in specific pathogen-free conditions in accordance with institutional guidelines of National Institute of Neuroscience, Tokyo, Japan. MOG_{35-55} (amino acid sequence, MEVGWYRSPFSRVVHLYRNGK) was synthesized at Toray Research Center (Tokyo, Japan). Incomplete Freund's adjuvant and heat-killed *mycobacterium tuberculosis* (H37Ra) were obtained from Difco Laboratories (Detroit, Michigan), and pertussis toxin was obtained from List Biological Laboratories (California). The hybridoma producing CEACAM1-specific antibody, AgB10,[33] was kindly provided by Nicole Beauchemin (McGill Cancer Center), and 293 EBNA cells transfected pCEP4-N-CEACAM-Fc, which produce a homophilic ligand of CEACAM1, CEACAM1-Fc fusion protein were kindly provided by Thomas M. Gallagher (Loyola University Medical Center).[34]

Induction and Evaluation of EAE

EAE was induced in mice as described previously.[35] Briefly, mice were immunized subcutaneously with 100 μg of MOG_{35-55} emulsified in incomplete Freund's adjuvant containing 500 μg of *M. tuberculosis*. Directly after the immunization and 48 hours later, mice were injected intraperitoneally with 200 ng of pertussis toxin. Clinical signs of EAE were assessed daily with a 0 to 6 scoring system (0, no signs; 1, partial loss of tail tonicity; 2, completely limp tail and abnormal gait; 3, partial hindlimb paralysis; 4, complete hindlimb paralysis; 5, fore- and hindlimb paralysis or moribund state; 6, dead).

Preparation of Antibody and Fusion Protein

The hybridomas producing AgB10 were cultured in a humidified atmosphere with 5% CO_2 at 37°C in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mmol/L L-glutamine, and 100 U/ml penicillin/streptomycin. The supernatants were collected and AgB10 was affinity-purified using a protein A column according to the manufacturer's instructions (Millipore, MA). 293 EBNA cells transfected pCEP4-*N*-CEACAM1-Fc were cultured in DMEM containing 10% heat-inactivated fetal calf serum, 2 mmol/L L-glutamine, and 100 U/ml penicillin/streptomycin. CEACAM1-Fc fusion protein was affinity-purified using protein G column from the collected supernatants (Amersham Bioscience, NJ).

MOG_{35-55} -Specific T Cell Response and Cytokine Assay

After immunization with MOG_{35-55}, mice were treated intraperitoneally with the indicated compounds, either 250 μg of AgB10 or 250 μg of control rat IgG antibody (Jackson Immuno Research, PA), or either 250 μg of CEACAM1-Fc fusion protein or 250 μg of a chimeric (mouse/human) anti-human CD20 mAb (rituximab) every second day from the day of immunization, day 0, to day 11. The animals were sacrificed at day 11 and inguinal and popliteal lymph nodes (LN) were sampled. Total LN cells were suspended in RPMI 1640 medium containing 2% syngeneic mouse serum, 2 mmol/L L-glutamine, 5×10^{-5} M/L 2-mercaptoethanol, and 100 U/ml penicillin/streptomycin, and were incubated in 96-well plates with 1×10^6 cells/well in the presence of 0, 1, 10, 30, or 100 mg/ml of MOG_{35-55}. Culture supernatant was collected 48 hours after stimulation, and IFN-γ and IL-17 in the

supernatant were determined by enzyme-linked immunosorbent assay (ELISA) using OptEIA kit (BD Bioscience, CA) and IL-17 ELISA kit (R&D systems), respectively.

Histology

Sixteen days after the immunization with MOG_{35-55}, the spinal cords were sampled and stored in 10% formaldehyde. Paraffin-embedded spinal cords were stained with either H&E or luxol fast blue.

Flow Cytometry

Liver mononuclear cells from B6 mice were isolated by Percoll density-gradient centrifugation. 1×10^6 cells/well were stimulated with 1 mg/ml plate-bound anti-CD3 mAb and 2.5 mg/ml Concanavalin A (ConA) in 96-well plates and collected for the use of flow cytometry. Cells were stained with α-galactosylceramide (α-GC) loaded dimeric mouse CD1 days followed by fluorescein isothiocyanate-conjugated AgB10, phycoerythrin-conjugated mAb A85-1, and allophycocyanin-conjugated anti-TCR β-chain. iNKT cells were gated as α-GC loaded CD1 days dimmer and TCRβ double-positive cells, and T cells were gated as TCRβ single-positive cells. Stained cells were analyzed using a FACSCalibur with CellQuest Software (Becton Dickinson, CA).

In Vivo *Injection of α-GC*

B6 mice were treated intraperitoneally with either 500 μg of AgB10 or 500 μg of control rat IgG antibody. Four days after the treatment, 250 μl of blood was collected at 2 or 6 hours after intravenous injection with 0.6 μg α-GC/dimethyl sulfoxide or control dimethyl sulfoxide. Blood samples were centrifuged at 3000 rpm for 30 minutes at 4°C, and serum was collected and IFN-γ and IL-4 were determined using ELISA kit (BD Bioscience, CA).

Statistics

EAE clinical scores for groups of mice are presented as the mean group clinical score ± SEM, and statistical differences were analyzed by the Mann-Whitney U nonparametric ranking test. Data for cytokines were analyzed with the two-way analysis of variance. In appropriate cases, post hoc comparisons were made.

Results

CEACAM1 Has a Role in Ameliorating EAE

To assess the role of CEACAM1 on EAE, we first examined the effect of CEACAM1-Fc fusion protein encoding the extracellular portion of the mCEACAM1-4L. CEACAM1-Fc fusion protein has been demonstrated to homophilically ligate the CEACAM1 molecule, which has been shown to inhibit IFN-γ production.[12] As shown in

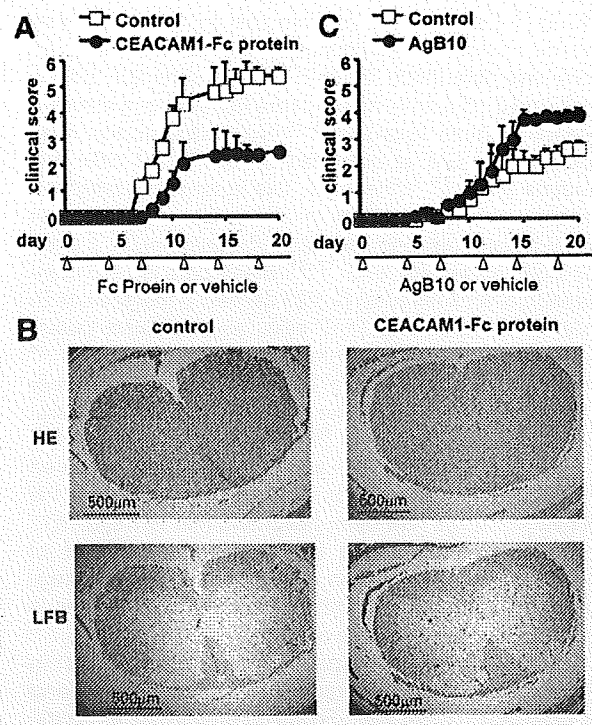

Figure 1. Effect of CEACAM1-Fc fusion protein or CEACAM1-specific antibody on MOG-induced EAE. EAE was induced in B6 mice by immunization with MOG_{35-55}. CEACAM1-Fc fusion protein (**A**) or a mAb for CEACAM1, AgB10 (**C**) was given twice per week starting from the day of immunization. **Arrowheads** indicate the time point of administration of CEACAM1 Fc fusion protein or AgB10. $^*P < 0.05$ vs. Control. The results represent the means ± SEM of eight mice per group. Representative data from two separate experiments is demonstrated. **B:** Histopathological assessment of the CNS region in EAE-induced mice. Shown are cellular infiltration and demyelination of the spinal cord of control or CEACAM1-Fc fusion protein-treated mice on day 16. Paraffin-embedded spinal cords were stained with H&E (**upper** panels) or luxol fast blue (LFB) (**lower** panels). Scale bar = 500 μm.

Figure 1A, administration of CEACAM1-Fc fusion protein significantly inhibited the development and the progression of EAE compared with control mice.

To characterize the immunosuppressive effect of CEACAM1, we performed the pathological analysis of CNS inflammation and demyelination in EAE-induced mice treated with CEACAM1-Fc fusion protein (Figure 1B). Histological examination of the spinal cord 16 days after EAE induction revealed less cellular infiltration and demyelination in CEACAM1-Fc fusion protein-treated mice, as compared with control mice.

We next examined the effects of CEACAM1 specific antibody, AgB10, on the development and progression of MOG_{35-55}-induced EAE in B6 mice (Figure 1C). Ligation of CEACAM1, either homophilically by CEACAM1-Fc fusion protein or heterophilically by microbial components such as the spike glycoprotein of murine hepatitis virus, has been demonstrated to inhibit the proliferation and cytokine production of T cells.[6–12] In contrast, AgB10 has been reported to enhance the T cell proliferation, indicating that AgB10 acts as a blocking antibody. As expected, the clinical scores of EAE were augmented in the mice treated with AgB10 compared with those of control mice.

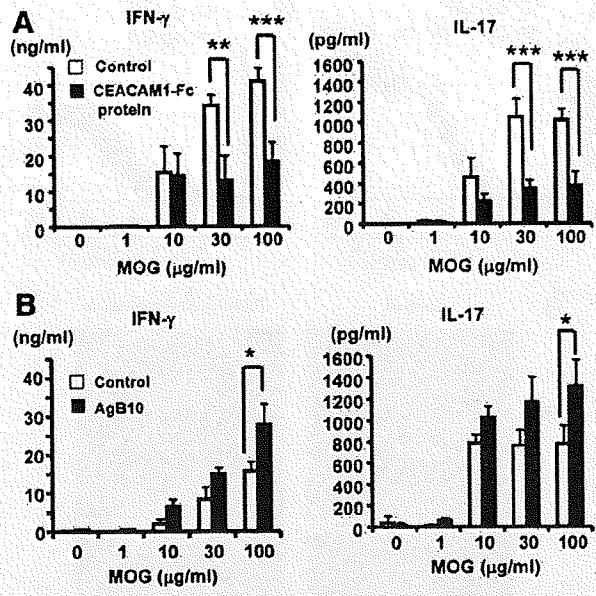

Figure 2. MOG_{35-55}-specific T cell responses in mice treated with CEACAM1-Fc fusion protein or with AgB10. B6 mice were treated with CEACAM1-Fc fusion protein (**A**) or AgB10 (**B**) twice per week from the day of immunization with MOG_{35-55}. Eleven days after the immunization, draining lymph node cells were incubated with MOG_{35-55}. Supernatants were collected from the culture and measured for the concentration of IFN-γ and IL-17 by ELISA. Data represent the mean ± SEM of samples from one of two independent experiments ($n = 3$ mice). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. Control.

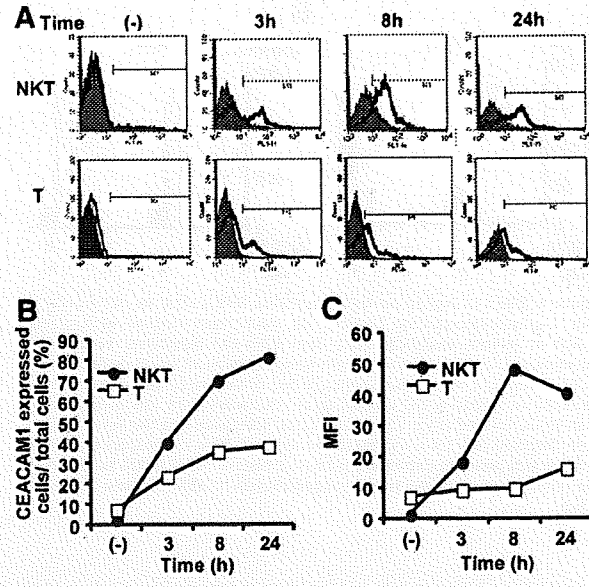

Figure 3. Expression of CEACAM1 on iNKT and T cells, after activation with ConA and anti-CD3 antibody. The histograms show the log fluorescence intensity of CEACAM1 on the surface of iNKT and T cells at the indicated time points after the activation with a combined treatment of ConA and plate bounded anti-CD3 antibody (**A**). The black curves indicate the fluorescence intensity of CEACAM1 on the surface of nontreated cells, and the gray silhouettes show the intensity of activated cells with ConA and anti-CD3 antibody. iNKT cells were gated as α-GC loaded CD1 dimmer and TCRβ double-positive cells, and T cells were gated as TCRβ-positive cells, respectively. The percentage of CEACAM1-expressing cells within total iNKT or T cells and mean fluorescence intensity of the expression at the indicated time points were shown in graph (**B**).

These results indicate that signals through CEACAM1 suppressed both the clinical and the pathological severity of EAE.

The Signal through CEACAM1 Reduces MOG_{35-55}-Specific IFN-γ and IL-17 Production

Since MOG_{35-55} induced EAE is thought to be mediated by MOG_{35-55}-specific Th1 and Th17 cells, we next examined MOG_{35-55}-specific T cell responses in CEACAM1-Fc fusion protein-treated (Figure 2A), or AgB10-treated mice (Figure 2B). We immunized mice with MOG_{35-55} and treated them with either AgB10 or CEACAM1-Fc fusion protein. Twelve days later, we harvested LN cells and re-stimulated them with MOG_{35-55} peptide *in vitro* to examine cytokine production and proliferation. Compared with cells from the control mice, LN cells obtained from CEACAM1-Fc fusion protein treated mice were significantly inhibited in IFN-γ and IL-17 production in responses to MOG_{35-55} re-stimulation (Figure 2A). IL-4 was not detected in the supernatant. On the other hand, *in vivo* treatment with AgB10 showed an enhancement of IFN-γ and IL-17 production in response to MOG_{35-55} stimulation (Figure 2B). Proliferative responses were not significantly different between control mice, CEACAM1-Fc protein-treated, or AgB10-treated mice (data not shown).

These results indicate that the suppressive effect of CEACAM1 on EAE was associated with reduction of MOG_{35-55}-specific IFN-γ and IL-17 production.

Rapid Expression of CEACAM1 on iNKT Cells after Activation

It has been reported that CEACAM1 is expressed on T cells early after activation, and its ligation directly inhibits IFN-γ production by such T cells. We therefore examined the time course of CEACAM1 expression by T cells *in vitro*. As reported previously, CEACAM1 expression was observed on T cells several hours after activation with ConA and anti-CD3 mAb *in vitro*. Moreover, we observed that there was a rapid and higher expression of CEACAM1 by CD1-restricted iNKT cells after activation (Figure 3A). The log fluorescence intensity of CEACAM1 on surface of iNKT and T cells and the percentage of CEACAM1 expressed cells within total iNKT or T cells showed a rapid and also enhanced expression of CEACAM1 on iNKT cells compared with T cells after activation (Figure 3B).

CEACAM1 Regulates IFN-γ Production from iNKT Cells

iNKT cells possess the ability to produce a wide variety of cytokines. Activation of iNKT cells is known to lead to either suppressive or stimulatory immune responses depending on the type of cytokine they produce.[30] We have demonstrated the rapid and enhanced expression of CEACAM1 specifically on iNKT cells (Figure 3A). Thus we next examined whether or not the administration of

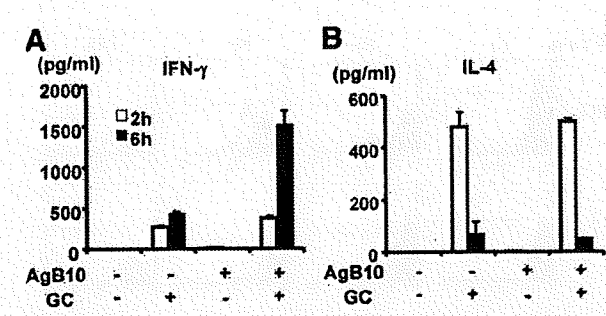

Figure 4. Effect of AgB10 on serum cytokine levels after *in vivo* injection with α-galactosylceramide (α-GC). B6 mice were treated with either AgB10 or control antibody. Four days after the treatment, serum was collected at 2 or 6 hours after intravenous injection of α-GC. Serum levels of IFN-γ and IL-4 were measured by ELISA. Increased levels of IFN-γ were observed in AgB10-α-GC treated mice compared with the control antibody-α-GC treated mice (**A**), whereas no alterations in the level of IL-4 were detected (**B**). Data represent the mean ± SEM of samples from one of three independent experiments ($n = 3$ mice). $^{***}P < 0.001$ vs. Control. The results represent the mean concentrations ± SEM of three mice per group.

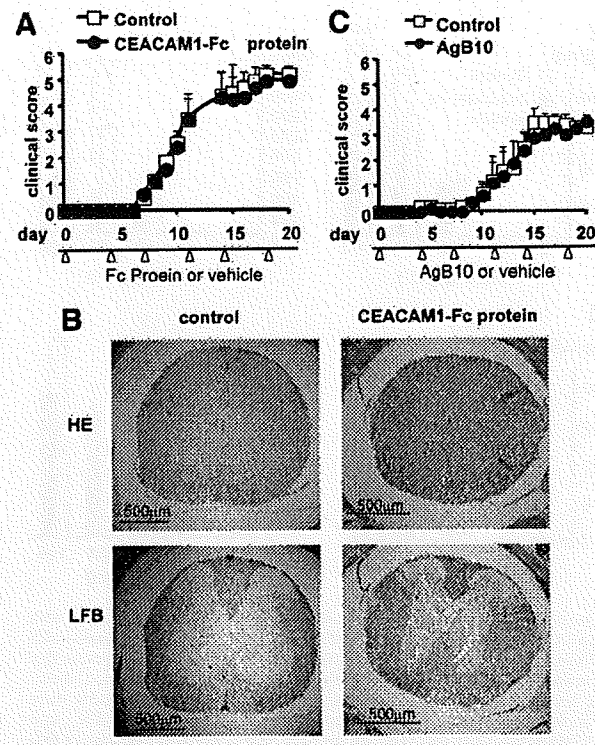

Figure 5. Effect of CEACAM1-Fc fusion protein or CEACAM1-specific antibody on EAE induced in Jα18$^{-/-}$ mice. EAE was induced in *Jα18*$^{-/-}$ mice by immunization with MOG_{35-55}. CEACAM1-Fc fusion protein (**A**) or AgB10 (**C**) was given twice per week starting from the day of immunization. **Arrowheads** indicate the time point of administration of CEACAM1-Fc fusion protein or AgB10. The results represent the means ± SEM of eight mice per group. Representative data from two separate experiments is demonstrated. **B:** Histopathological assessment of the CNS region in *Jα18*$^{-/-}$ mice induced with EAE. Shown are cellular infiltration and demyelination of the spinal cord of control or CEACAM1-Fc fusion protein-treated mice on day 16. Paraffin-embedded spinal cords were stained with H&E (**upper** panel) or LFB (**lower** panels). Scale bar = 500 μm.

AgB10 has an effect on cytokine production by iNKT cells. Mice were injected intravenously with iNKT cell-specific ligand, α-GC, or vehicle, and serum levels of IFN-γ and IL-4 were measured. Mice pretreated with AgB10 and injected with α-GC showed significantly increased level of IFN-γ, as compared with mice treated with control antibody and injected with α-GC (Figure 4A). No significant difference was observed in IL-4 production (Figure 4B). The level of IL-12 in serum was not altered in AgB10-treated mice, and IL-17, IL-21, or IL-23 were not detected in the serum (data not shown). The results suggest that the signal from CEACAM1 have a role in IFN-γ production by iNKT cells.

The Modulation of EAE by CEACAM1 Was Abrogated in iNKT Cell-Deficient Jα18 Mice

Since iNKT cells highly express CEACAM1 after activation, it was of interest to investigate whether the iNKT cells are involved in CEACAM1-mediated amelioration of EAE. To address this question, we examined the effect of CEACAM1-Fc fusion protein on the development of MOG_{35-55}-induced EAE in *Jα18*$^{-/-}$ mice, which genetically lack iNKT cells. In contrast to B6 mice, no alteration in the severity of EAE was observed in CEACAM1-Fc fusion protein treated *Jα18*$^{-/-}$ mice, as compared with control mice (Figure 5A). To further determine the effect of the ligation of CEACAM1 on EAE in *Jα18*$^{-/-}$ mice, we analyzed the CNS inflammation and demyelination in EAE-induced *Jα18*$^{-/-}$ mice treated with CEACAM1-Fc fusion protein. In contrast to wild-type B6 mice, histological examination of the spinal cord of *Jα18*$^{-/-}$ mice showed cellular infiltration and demyelination to a similar extent as sham-treated mice (Figure 5B). We next induced EAE in *Jα18*$^{-/-}$ mice treated with either AgB10 or control antibody. Again, no suppression of clinical EAE was observed in AgB10- treated *Jα18*$^{-/-}$ mice, as compared with the control mice (Figure 5C).

These data show that CEACAM1 signal modulation does not affect on the severity of clinical and pathological EAE in mice lacking iNKT cells.

The Modulation of MOG_{35-55}-Specific IFN-γ and IL-17 Production by CEACAM1 Required iNKT Cells

The suppression of EAE by the ligation of CECAM1 in B6 mice was associated with a reduction in MOG_{35-55}-specific IFN-γ and IL-17 production. We next examined MOG_{35-55}-specific T cell responses in CEACAM1-Fc fusion protein-treated (Figure 6A), or AgB10-treated *Jα18*$^{-/-}$ mice (Figure 6B) by *ex vivo* re-challenge with MOG_{35-55} on day 11 after the immunization of MOG_{35-55}. In contrast to B6 mice, LN cells from CEACAM1-Fc fusion protein-treated *Jα18*$^{-/-}$ mice exhibited no significant reduction of MOG_{35-55} specific IFN-γ and IL-17 production compared with the control mice (Figure 6A). Additionally, *in vivo* treatment of *Jα18*$^{-/-}$ mice with AgB10 also did not significantly enhance of MOG_{35-55}-specific T cell IFN-γ and IL-17 production (Figure 6B).

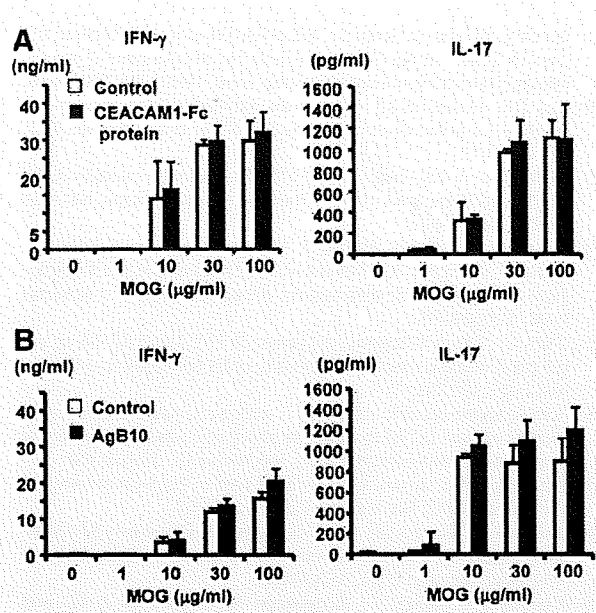

Figure 6. MOG_{35-55}-specific T cell responses in $J\alpha18^{-/-}$ mice treated with CEACAM1-Fc fusion protein or with AgB10. $J\alpha18^{-/-}$ mice were treated with CEACAM1-Fc fusion protein (**A**) or AgB10 (**B**) twice per week from the day of immunization with MOG_{35-55}. Eleven days after the immunization, draining lymph node cells were incubated with MOG_{35-55}. Supernatants were collected from the culture and measured for the concentration of IFN-γ and IL-17 by ELISA. Data represent the mean ± SEM of samples from one of two independent experiments (n = 3 mice).

These results indicate that iNKT cells play an important role in CEACAM1-mediated reduction of MOG-specific IFN-γ and IL-17 production.

Discussion

The present study demonstrated that the signal through CEACAM1 suppressed EAE in association with a reduction in MOG_{35-55}-specific production of IFN-γ and IL-17. Moreover, we showed that CEACAM1 was expressed at an early time point by iNKT cells after activation and CEACAM1 also affected the cytokine production by iNKT cells, including IFN-γ, but not IL-4. Finally, we demonstrated that CEACAM1-mediated modulation of EAE and MOG_{35-55}-specific cytokine production required iNKT cells.

Since both IFN-γ and IL-17 are known as potent inducers of EAE,[21,27–29] CEACAM1-mediated reduction of these cytokines is thought to have a significant role in ameliorating EAE. Although the mechanisms of IFN-γ and IL-17 reduction in CEACAM1-mediated suppression of EAE are not clearly defined so far, we found that the effects of AgB10 and CEACAM1-Fc fusion proteins on EAE and MOG_{35-55}-reactive cytokine responses were abolished in iNKT cell-deficient $J\alpha18^{-/-}$ mice. Thus we concluded that CEACAM1-mediated suppression of EAE was mediated via iNKT cells. Activation of iNKT cells are known to modulate dendritic cell functions, and Kammerer et al reported that AgB10 triggered release of IL-12 from dendritic cells and facilitated priming of naive $CD4^+$ T cells with a Th1-like phenotype.[36] In contrast, Iijima et al showed that CEACAM1-mediated inhibition of Th1-mediated colitis was not dependent on the modulation of IL-12, consistent with this finding, IL-12 was not affected in EAE-induced mice by the *in vivo* treatment of AgB10. Since iNKT cells have been shown to produce IL-21, which promotes the development of Th17 cells,[37] CEACAM1 expression by iNKT cells may have a regulatory role in IL-17 production by Th17 cells via IL-21. However, the production of IL-21 upon iNKT cell activation was not altered by treatment with AgB10. In addition, production of IL-23, which promotes Th17 cell maintenance by activated iNKT cells was not altered in mice treated with AgB10, as compared with control mice. Therefore, the mechanisms how CEACAM1-treated iNKT cells modulate MOG_{35-55} reactive Th1 and Th17 cells remain to be elucidated.

Recently, Mars et al reported that activation of iNKT cells with α-GarCer during priming of the $CD4^+$ T cell response prevents the differentiation of naïve $CD4^+$ T cells toward the Th17 lineage, and the cytokine neutralization experiments indicated that IL-4, IL-10, and IFN-γ are involved in the iNKT cell-mediated regulation of T cell lineage development.[38] Although the direct mechanisms of iNKT cells in regulating the Th17 compartment are still in question, iNKT cells were shown to have a regulatory role in development of the Th17 lineage. Our laboratory reported that antibiotic treatment alters the composition of gut flora, resulting in amelioration of EAE in a iNKT cell-dependent manner.[39] iNKT cell-dependent amelioration of EAE was associated with the suppression of MOG_{35-55}- reactive Th17 cells, although the mechanism by which iNKT cells modulate MOG_{35-55}-reactive Th17 cells remained unclear. It was speculated that altering the compositions of gut flora by antibiotic treatment critically influences the function of iNKT cells, which resulted in a reduction of MOG_{35-55}-reactive Th17 cells. Since various bacterial and viral pathogens *trans*-ligate CEACAM1 and suppresses the activation and proliferation of T cells, it is possible that the alteration of cytokine production in physiological or pathological conditions is partly dependent on the way of *trans*-ligation of pathogens and CEACAM1 on iNKT cells.[3,12,40–45]

In conclusion, this study demonstrates for the first time that CEACAM1 negatively regulates the severity of EAE via an iNKT cell-dependent mechanism. Considering that the selective induction of cytokines by iNKT cells by synthetic ligands has been reported to suppress EAE,[32,46] CEACAM1 may prove to be a novel target for immunotherapy of multiple sclerosis.

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Back to the future for multiple sclerosis therapy: focus on current and emerging disease-modifying therapeutic strategies

The last decade has seen numerous advances in the treatment of multiple sclerosis with six immunotherapeutic agents licensed for use. Although these therapeutic agents have powerful effects upon the inflammatory phase of disease, they have limitations in treating the progression of disability and in their safety profile. This review focuses on our current understanding of first- and second-line treatments for multiple sclerosis, including combination therapies, and also discusses the most promising novel therapeutic strategies on the horizon. Such agents include orally administered immunosuppressive drugs, monoclonal antibodies, antigen-specific tolerance, and neural protection and repair strategies. The challenge ahead lies in the delivery of potent drugs to inhibit inflammation and neurodegeneration while limiting side effects. Further elucidation of the pathophysiology of disease may provide new clinical targets and disease-relevant biomarkers that, in combination with proteomics, may help personalize treatment to individual patients.

KEYWORDS: clinical trial » glatiramer acetate » IFN-β » immunosuppression » immunotherapy » multiple sclerosis » natalizumab » statin

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Multiple sclerosis (MS) is the most common human demyelinating disease of the CNS affecting at least a few million people in the world. Inflammation in the CNS causes the destruction of the myelin surrounding axons and also oligodendrocytes, the myelin-forming cells of the CNS, which leads to the dysfunction of axonal conduction. Symptoms of MS patients vary greatly depending on the location of white matter lesions and often lead to severe physical and cognitive disability. Diagnosis of MS previously relied on clinical examination assisted by laboratory examinations, including the assessment of oligoclonal immunoglobulin bands in cerebrospinal fluid (CSF). More recently, MRI plays a crucial role in diagnosing MS in the clinic as it can visualize most CNS lesions in MS [1]. MS has a number of subtypes based on the temporal character of the disease. Relapsing–remitting MS (RR-MS) is the most common form, present in two-thirds of MS patients, and presents as periods of disease episodes (relapses) followed by periods of remission. Within 10 years after onset, a significant proportion of RR-MS patients start to exhibit progressive worsening of disease without showing relapses or remission. Such patients are diagnosed as having secondary progressive MS (SP-MS). The likelihood that RR-MS may develop into SP-MS is significantly reduced if the patients are given disease-modifying agents such as IFN-β. Primary progressive MS (PP-MS) is a progressive form of disease present in 5–10% of MS patients that is refractory to currently available treatment. PP-MS patients are at a high risk of becoming neurologically disabled only a few years after onset. Although the pathogenesis of MS is unknown, there are a number of risk modifiers, including genetic susceptibility [2,3] and environmental factors such as infectious pathogens [4]. MS is thought to have an autoimmune pathology based on the presence of activated myelin-specific T cells in the peripheral blood of MS patients and the fact that immunization with myelin antigens or injection of myelin-specific T cells can induce a CNS demyelinating disease, experimental autoimmune encephalomyelitis (EAE), in animals. Therefore, most therapeutic agents have been directed towards blocking or inhibiting the function and activation of the immune response, especially the T cell.

Current MS treatment

Treatment of MS is usually either symptomatic or immunomodulatory (disease modifying). Treatments for annoying symptoms, such as pain, numbness and bladder dysfunction, are important in improving the quality of life of MS patients. In this review, we will concentrate on the six disease-modifying treatments licensed by the US FDA for the treatment of RR-MS. The development and application of therapeutic agents has largely been pioneered by preclinical studies in animal models, which have provided an understanding of disease

processes and have identified targets for treatment. Thus, animal models of MS, such as EAE, are essential for testing and developing new therapeutic strategies.

Based on data from animal studies and the presence of large numbers of inflammatory cells in the CNS lesions of MS patients, many of the pioneering treatments for MS were directed towards anti-inflammatory strategies. Originally, these were broad-based immunosuppressive agents that could dampen inflammatory responses nonspecifically. As such, glucocorticoids with potent anti-inflammatory and immunosuppressive properties were among the first agents to be effective in treating acute episodes of MS, decreasing the duration of relapse periods. Steroids, such as methylprednisolone, are generally administered intravenously as a bolus of 0.5–1 g daily for 3–5 days during relapse episodes. The mechanisms of action of glucocorticoids include inhibition of T-cell cytokine release, increase in T-cell apoptosis and anti-inflammatory effects upon the blood–brain barrier (BBB) [5]. Although glucocorticoids are a standard treatment for limiting the duration of relapse episodes, they appear to have little effect on the number of relapses experienced and the patients' long-term progression to disability. Other immunosuppressive drugs used in the prevention of organ transplant rejection, such as cyclophosphamide, cyclosporine, azathioprine and methotrexate, have also demonstrated efficacy in modulating MS disease activity [7–9]. However, these agents are limited by toxicity and their nonspecific nature of immunosuppression. Nevertheless, their use provided an important step towards developing new immunosuppressive agents with fewer side effects, which could be targeted to more specific branches of the immune system as discussed later in this review.

When reviewing data from MS clinical trials, it is important to consider the 'placebo effect'. In some cases, patients receiving placebo can show similar or improved effects compared with those from patients on the active drug treatment. The scientific basis of this effect is not clear but may be linked to neurological and hormonal changes that take place when a patient believes the placebo is a drug that can cure their illness. Although this effect is common in many clinical trials of different diseases, it must be particularly regarded in MS, as the symptoms of MS can remit at any time by unknown mechanisms, and therefore give a misleading view of the effectiveness of a drug or placebo treatment.

Although none of the following immunomodulatory agents are a cure, they nevertheless provide some benefit for the treatment of MS by reducing the number and severity of clinical relapse attacks and the number of new lesions as measured by MRI, which would lower the neurological disability in the treated patients. It is noteworthy that 'responder' patients can be free from neurological impairment over 10 years after starting treatment, whereas nonresponder patients seem to have no clinical benefits from the treatment.

IFN-β

Despite many therapeutic claims, the first agent to show a significant therapeutic benefit in double-blind, Phase III, placebo-controlled trials was IFN-β, a pleiotropic cytokine produced by fibroblasts with antiviral, immunomodulatory and antineoplastic properties. In addition to inhibiting T-cell proliferation, IFN-β may suppress the production of proinflammatory cytokines and enhance IL-10 production. It has also been shown to improve the integrity of the BBB, thereby inhibiting immune cell migration into the CNS [10–12]. IFN-β was also demonstrated to have protective effects in EAE when delivered by gene therapy [13].

There are currently three different recombinant IFN-β preparations in use for MS. IFN-β_{1b} (marketed as Betaferon® in Europe and Betaseron® in the USA) is an unglycosylated recombinant protein produced in *Escherichia coli* and modified from the native protein by substitution of L-cysteine by L-serine at position 17 to provide improved *in vivo* stability [14]. The principal IFN-β_{1b} clinical trial administered the drug to 372 RR-MS patients randomized to receive placebo or a low dose (1.6 million international units [MIU]) or high dose (8.0 MIU) of IFN-β_{1b} subcutaneously every other day [15]. The high-dose group experienced a significantly reduced exacerbation rate per year (34% lower) and a 58% decrease in active lesions per year but no effect on disability progression. This study was the first to show decreased MRI activity in conjunction with positive treatment. Betaseron was the first disease-modifying therapy approved for treatment of RR-MS by the US FDA, in 1993.

IFN-β_{1a} (Avonex® and Rebif®) is a glycosylated recombinant IFN-β produced in Chinese hamster ovary cells that is identical to human native IFN-β [16]. A randomized, double-blind, placebo-controlled trial of Avonex in 301 RR-MS patients demonstrated that 30 μg administered intramuscularly once a week could reduce the

relapse rate by 32% and new enhancing lesions by 50% [17]. In addition, patients experienced a 37% reduction in the probability to disease progression as measured by expanded disability status scale (EDSS). A Phase III clinical trial with Rebif randomized 560 patients to receive either placebo or 22 or 44 µg IFN-β_{1a} subcutaneously three-times a week [18]. The primary outcome was a reduction in the number of relapse episodes, which were significantly reduced compared with placebo (33 and 37% in the 22- and 44-µg dose groups, respectively). MRI-active lesions were reduced in the low-dose (67%) and high-dose group (78%). In addition, the time to confirmed progression of disability was delayed in the two IFN-β-treated groups (risk ratio of 0.68 and 0.62 in the 22- and 44-µg dose groups, respectively).

Avonex has been licensed by the FDA for the treatment of RR-MS to decrease both the rate and severity of relapse episodes in the USA since 1996 and Europe since 1998. Rebif has been licensed as a treatment for RR-MS to decrease relapse rates and delay the accumulation of disability since 1998 in Europe and 2002 in the USA. Side effects of IFN-β therapy usually appear between 2 and 8 h postadministration, last up to 24 h and include influenza-like symptoms (in 75% of IFN-β_{1b} and 50% of IFN-β_{1a} patients), such as muscular aches, fever and headache [19–21]. Symptoms usually self-resolve. Injection-site inflammation can also occur in approximately 85–92% of patients treated subcutaneously, but decreases over time [20,21].

One potential problem with long-term IFN-β treatment is the induction of IFN-β-neutralizing antibodies that may be partially responsible for the difference in efficacy observed between treated individuals [22]. This could potentially limit future administration of these drugs. There is a suggestion that there is an increase in neutralizing antibodies to IFN-β_{1b} compared with IFN-β_{1a}, which may be due to its increased stability *in vivo*. Alternatively, foreign products used in its preparation may cause it to be immunogenic. Therefore, a recent study using a new formulation of Rebif (Rebif New Formulation [RNF]), produced in the absence of fetal bovine serum and human serum albumin used as excipients or carrier for the active drug, was undertaken with the aim of improving injection tolerability and reducing immunogenicity. A Phase IIIb, open-label study demonstrated that RNF administered at 44 µg subcutaneously three-times weekly had a significantly improved safety and immunogenic profile compared with the original Rebif formulation [23].

Glatiramer acetate

Glatiramer acetate (GA; Copaxone®) is a synthetic chemical composed from random polymers of four amino acids (glutamic acid, lysine, alanine and tyrosine) and was designed to mimic myelin basic protein (MBP), one of many potential encephalitogenic proteins in MS. Although it was designed to induce animal models of MS, it was found to effectively inhibit the disease course [24–26]. Therefore, it was tested in human MS trials. A double-blind, randomized, placebo-controlled Phase III study to investigate the effect of GA on a change in relapse rate treated 251 RR-MS patients with GA 20 mg subcutaneously [27]. After 2 years, treatment patients receiving GA had a relapse rate of 1.19 ± 0.13 compared with 1.68 ± 0.13 in the placebo group, a 29% reduction ($p = 0.007$).

Another randomized, double-blind, placebo-controlled study treated 239 RR-MS patients over 9 months with either GA 20 mg subcutaneously or placebo, and demonstrated that GA treatment causes a significant reduction in the total number of enhancing lesions versus placebo ($p = 0.003$). In addition, GA treatment also significantly reduced the number of new enhancing lesions ($p < 0.003$), the monthly change in the volume of enhancing lesions ($p = 0.01$) and reduced the relapse rate by 33% ($p = 0.012$) [28]. It was approved for the treatment of RR-MS in the USA by the FDA in 1997 and is also licensed in Canada and most of Europe. GA is self-administered subcutaneously by the patient in doses of 20 mg. A double-blind trial to study the safety and efficacy of a higher daily dose of GA (40 mg subcutaneously) compared with the approved dose of 20 mg subcutaneously was undertaken in RR-MS patients over 9 months [29]. The study observed that patients receiving GA 40 mg subcutaneously had a lower risk for cumulative number of new gadolinium-enhancing lesions compared with the GA 20 mg subcutaneously group, although this did not reach statistical significance. However, the numbers of active enhancing MRI lesions in months 7, 8 and 9 were reduced in the high-dose and low-dose group (75 and 65%, respectively) compared with baseline measurements. In addition, a significant increase in time to the first relapse was observed in the high-dose group of 213 days compared with 80 days in the low-dose group.

Recently, a 14-month, double-blind, randomized, multicenter trial compared the effects of two doses (5 or 50 mg) of GA delivered orally once daily to 1644 RR-MS patients [30]. Unfortunately, the primary end point of number of relapses or secondary end point of MRI lesions was negative in all groups.

The mechanism of action of GA is currently unclear but evidence from MS and EAE studies suggest 'bystander suppression', whereby treatment can alter T-cell antigen reactivity by inducing GA-reactive $CD4^+$ Th2 cells, which can suppress Th1-type inflammation [31–35]. In addition, studies have demonstrated that GA may induce a suppressor-cell subset, such as $CD4^+CD25^+Foxp3^+$ cells, or act as an alternative ligand and induce tolerance of the aggressive encephalitogenic T-cell response in MS [36–39]. Further studies have observed the induction of a GA-specific $CD8^+$ cytotoxic/suppressor cell subset in GA-treated MS patients [40–42]. Whereas there is a deficit of $CD8^+$ T-cell-mediated suppression in untreated MS patients [43], GA treatment can enhance the numbers of GA-reactive $CD8^+$ T cells [42]. Furthermore, the activation of GA-reactive $CD8^+$ cytotoxic T cells by GA leads to the HLA class I-restricted suppression of $CD4^+$ T cells, especially when in the activated state [42]. Therefore, GA-restricted $CD8^+$ T cells may provide benefit in MS patients undergoing GA treatment by suppressing encephalitogenic $CD4^+$ T cells involved in the pathogenesis of MS.

A study in EAE proposed that GA may be neuroprotective and could augment neurogenesis by inducing the proliferation of oligodendrocyte progenitor cells [44,45]. Side effects from GA are generally mild and well tolerated by patients. The most common of these (10–15% of GA treated patients) is a reaction at the injection site, which may cause inflammation or itching. In addition, more unpleasant reactions include chest tightness, palpitations, weakness, nausea, flushing, anxiety and muscle stiffness, which can occur a short while after administration but usually resolve a few minutes later.

Mitoxantrone

Mitoxantrone (Novantrone®) is a type II topoisomerase inhibitor used as an antineoplastic agent in certain forms of cancer, such as metastatic breast cancer. Its mechanism of action is as a cytotoxic agent that causes disruption to DNA synthesis and repair. It is this ability to suppress the rapidly expanding encephalitogenic cells of the immune system that suggested its use in MS. It has also been demonstrated to have a number of immunosuppressive functions, including the inhibition of Th1-type cytokines IFN-γ, TNF-α and IL-2; suppression of proliferation of B cells, T cells and macrophages and immune cell trafficking [46–49]. A randomized but open-label clinical trial in 42 patients with very active MS treated patients with an infusion containing mitoxantrone 20 mg and methylprednisolone 1 g or methylprednisolone 1 g alone for 6 months to determine the effect upon the percentage of patients without new active MRI lesions [50]. At the beginning of the study, the mitoxantrone plus methylprednisolone group was 10% and methylprednisolone alone was 4.8%. Following 6 months of treatment, the percentages were 90 and 31% for the mitoxantrone plus methylprednisolone group and methylprednisolone alone group, respectively ($p < 0.001$). A second study in Italy tested the efficacy of mitoxantrone (8 mg/m^2 body surface) in 51 randomized RR-MS patients to affect the number of patients experiencing clinical disability progression as measured by blinded physicians on the EDSS [51]. Following 24 months of treatment, 7% (two of 27) of mitoxantrone and 37% (nine of 24) of placebo-treated patients deteriorated by one point on the EDSS ($p = 0.02$). Third, a double-blind trial in SP-MS ($n = 49$) compared mitoxantrone (12 mg/m^2 body surface) versus methylprednisolone (1 g) administered as an infusion 13-times over 32 months. The study demonstrated a significant improvement in the mitoxantrone-treated group regarding the EDSS and a decrease in the total number of gadolinium-enhancing lesions [52]. Mitoxantrone has been licensed for worsening forms of relapsing MS by the FDA since 2000, as described later. As mitoxantrone has a long half-life, it can be administered to patients either monthly or every 3 months as an intravenous infusion at doses between 10 and 20 mg (6–12 mg/m^2). In a Phase III trial, low- and medium-dose mitoxantrone (5–12 mg/m^2) were well tolerated but the greatest efficacy was seen at higher doses [53]. However, cumulative doses above 140 mg/m^2 can cause cardiotoxicity, which may also limit the length of treatment available to patients. It is often used in patients with high disease activity, with numerous relapse episodes and increasing disability, over a course of 2–3 years in an effort to stabilize disease. Recently, it has been used in patients who still suffer relapses despite being on other treatments, such as copaxone or IFN-β. Side effects of mitoxantrone include cardiomyopathy (at doses above 140 mg/m^2), nausea, hair thinning, infertility and decreased white blood cell count.

Natalizumab

Natalizumab (Tysabri®) is a humanized monoclonal antibody against α4-integrin, a cell-surface adhesion molecule expressed on hematopoietic cells, which upon binding to its ligand,

VCAM-1, allows extravasation of inflammatory cells into the CNS. Therefore, the mechanism of action of natalizumab is to block the adhesive interaction between encephalitogenic immune cells and the endothelial cells of the BBB, thus preventing their access to the CNS and causing inflammation. A Phase III study treated 942 RR-MS patients with natalizumab 300 mg intravenously every 28 days for up to 28 months or placebo to determine the effect of natalizumab on the number of new gadolinium-enhancing lesions. No new lesions were observed in 96% of natalizumab-treated patients and 68% of the placebo group [54]. In addition, natalizumab was demonstrated to be very effective at inhibiting relapse rates by 68% at year 1 ($p < 0.001$) and slowing the progression of disability (42% over 2 years; $p < 0.001$). A randomized, double-blind, placebo-controlled trial in 213 RR-MS or SP-MS patients to test two doses of natalizumab (3 or 6 mg natalizumab per kg bodyweight) demonstrated reductions in the mean number of new gadolinium-enhancing lesions 1.1 ($p < 0.001$), 0.7 ($p < 0.001$) and 9.6 in the 6 mg, 3 mg and placebo groups, respectively [55]. In addition, fewer patients experienced relapses 18.9% ($p = 0.02$), 19.11% ($p = 0.002$) and 38% of patients in the 6 mg, 3 mg and placebo groups, respectively.

Natalizumab is administered every 28 days by intravenous infusion. Adverse events of natalizumab treatment include fatigue, headache and nausea. Natalizumab was originally approved for use in MS by the FDA in 2004, but was withdrawn in 2004 after progressive multifocal leukoencephalopathy (a rare viral demyelinating disease of the CNS induced by the John Cunningham (JC) virus, often observed in people with immune deficiencies) was observed in patients taking natalizumab and IFN-β_{1a} in combination, as discussed later. After a safety review in 2006, natalizumab was relicensed for use in RR-MS as a monotherapy to delay the onset of relapses and decrease the accumulation of disability by the FDA and by the EMEA. However, two deaths have recently been recorded following natalizumab monotherapy and, therefore, further pharmacovigilance is required in the future use of natalizumab [201].

Therapies for progressive forms of MS

Although a number of partially effective treatments exist for the RR-MS form of disease, there are no licensed disease-modifying therapies for PP-MS. Therapeutic studies in PP-MS have been limited owing to patient numbers (~10% of all MS patients) and also the difficulty in specific diagnosis. Despite this, there have been a number of PP-MS clinical trials. These include randomized, controlled trials with immunosuppressive agents such as cyclosporine A [7], azathioprine [56,57], methotrexate [9] and cladribine [58], all of which demonstrated no significant treatment effect.

Although a double-blind, placebo-controlled clinical trial of mitoxantrone in 61 PP-MS patients also demonstrated no effect upon disease progression [59], it demonstrated a higher efficacy when tested in a double-blind, placebo-controlled clinical trial in SP-MS patients. Similar to the trials in RR-MS, it significantly reduced the number of relapses in SP-MS patients (adjusted total number of treated relapses: 0.38; $p = 0.0002$) and had some effect on the change in the EDSS (change in expanded disability status scale: 0.24; $p = 0.0194$) [60]. The higher efficacy of mitoxantrone compared with other immunosuppressive agents may be due to its sequestration in the body's tissues and slow release, subjecting immune cells to long-term suppression, although the superiority of mitoxantrone above other immunosuppressive drugs is not clear as there are no comparable studies as yet. Mitoxantrone is now approved by the FDA for reducing the progression of disability and frequency of relapses in SP-MS patients or those with worsening RR-MS whose disease is abnormal between relapses.

Glatiramer acetate has been demonstrated to reduce relapse rates in RR-MS. Results have recently been published on the largest trial in PP-MS to date, a double-blind, placebo-controlled clinical trial in 943 patients [61]. Patients were administered GA subcutaneously at a dose of 20 mg daily with primary outcome measurements consisting of a change in EDSS. Secondary outcomes included brain lesion loads as measured by MRI and brain atrophy. Although there was a trend toward a delay in time to sustained accumulated disability, this was not significant compared with the placebo group (hazard ratio: 0.87; $p = 0.1753$). However, there were significant reductions in MRI-enhancing lesions in the first year of treatment ($p = 0.0022$). Unfortunately, the clinical trial was terminated early owing to an interim analysis study indicating that no treatment effect was observed in this study.

As IFN-β had been shown to be effective in the RR-MS form of disease, a number of clinical trials have tested IFN-β therapy in SP-MS.

However, IFN-β therapy in the progressive forms of MS has generally been less favorable. The Secondary Progressive Efficacy Trial of IFN-β_{1a} in Multiple Sclerosis Study (SPECTRIMS) demonstrated that IFN-β_{1a} or placebo administered subcutaneously at 22 or 44 µg to SP-MS patients three-times a week for 3 years had no effect on disability progression using EDSS, but did have some small benefit on reducing relapse episodes (relapse episodes per year: 0.71–0.50 for both doses; $p < 0.001$) [62,63]. Clinical trials by the Nordic Secondary Progressive Multiple Sclerosis study and the International Multiple Sclerosis Secondary Progressive AVONEX Clinical Trial (IMPACT) demonstrated no effect of IFN-β_{1a} on time to progression of disability by subcutaneously or intramuscular injection although there was a significant effect on the number of relapses in the IMPACT study [64,65].

A clinical trial to investigate IFN-β_{1b} (Betaseron) treated 718 SP-MS patients with 8 MIU of IFN-β_{1b} or placebo subcutaneously every other day for 2 years demonstrated that 21.7% of patients treated with IFN-β_{1b} experienced reduced progression to disability compared with control groups. In addition, they measured a reduction in the incidence of clinical relapses and new MRI lesion formation [66]. A trial by The North American IFN-β study used 939 patients with SP-MS treated subcutaneously with IFN-β_{1b} (Betaseron) or placebo every other day for 3 years. There was no difference in time to progression disability between the treatment groups and the trial was terminated early [67].

The first study of IFN-β in PP-MS was an exploratory, randomized, controlled trial in 50 patients who received IFN-β_{1a} weekly in either 30 or 60 µg doses intramuscularly over 2 years [68]. The primary outcome was the time to sustained progression of disability using the EDSS, and secondary outcomes included MRI lesion load, cerebral and spinal cord atrophy. The 30-µg dose was well tolerated and demonstrated significant effects in reducing the lesion load. By contrast, the 60-µg dose was not well tolerated and resulted in an increase in liver enzymes, influenza-like symptoms and a trend toward worse CNS atrophy. Neither dose had an effect upon the primary outcome [68]. A double-blind, placebo-controlled, trial to test the efficacy of IFN-β_{1b} in 49 patients with PP-MS was undertaken treating patients subcutaneously with 8 MIU on alternate days for 2 years. Although IFN-β_{1b} had a significant effect on lesion load, there was no effect on disease progression [69].

In conclusion, clinical trials in the RR-MS, SP-MS and PP-MS forms of MS demonstrate that immunomodulation of disease can be effective in earlier stages of disease when the inflammatory component of disease plays a major role in the pathology of MS, as evidenced by fresh MRI lesions in the CNS and clinical relapses. However, once disease advances to the progressive forms of disease, where brain atrophy and axonal degeneration occur, potentially independent of MRI lesion formation, then immunomodulatory agents appear to be relatively ineffective. Further knowledge of the underlying pathology in PP-MS is vital to future clinical trials for this subtype of disease.

Future treatment strategies

Although IFN-β and GA form the first-line therapies for MS owing to their modest disease-modifying effects and safety profile, there is currently no effective treatment available to reduce the long-term disease progression. Mitoxantrone can be considered for patients with worsening RR-MS and natalizumab as a second-line treatment for those patients with aggressive RR-MS who do not respond to the other therapies. Together, these treatments can be effective in reducing new MRI lesions and relapse rates; however, their effects on the underlying progressive neurodegeneration and prevention of the transition between RR-MS and SP-MS/PP-MS is currently unclear. Therefore, there is a real need to develop new therapies that can halt or significantly retard the disease process. Studies of combination therapy of existing treatments hope to increase efficacy by synergy while limiting side effects. Furthermore, neuroprotective or restorative strategies may prevent or repair damage in the CNS and, therefore, could potentially restore functions lost due to axonal degeneration. This type of research has yielded a number of recent clinical trials to study novel therapies for MS treatment. Here, we discuss some of the promising new therapeutic strategies for MS. The current and future therapeutic targets and their proposed mechanisms of action for MS therapy are presented in Figure 1.

Combination therapies

Although GA and IFN-β have demonstrated partial efficacy in treating MS, a number of patients remain low or nonresponders. Usually, in these instances, patients are transferred to second-line treatments, such as mitoxantrone or natalizumab, although these can have some safety issues. Therefore, one method to enhance