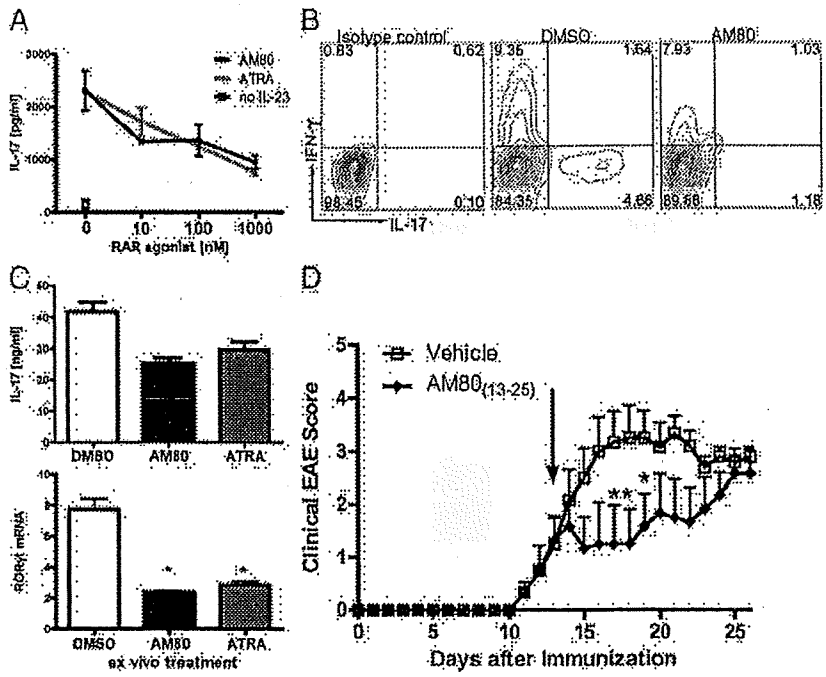

Figure 4. AM80 suppresses IL-17 production by differentiated Th17 cells and ameliorates EAE in therapeutic intervention. A: $CD4^+CD44^+CD25^-CD62L^{low}$ memory T cells were stimulated by plate-bound anti-CD3 antibody for 4 days in the presence of IL-23. Dose-dependent decrease of IL-17 production by RAR agonists was shown in left panel. Intracellular cytokine staining of TCR^+CD4^+ cells shows decreased percentage of $IL\text{-}17^+$ memory T cells after treatment with 100 nmol/L AM80 (B). Graphs are representative of two independent experiments. C: CNS-infiltrating T cells from mice with severe EAE (score 3–4) were isolated and restimulated with immobilized anti-CD3 in the presence of RAR agonists (100 nmol/L) for 3 days. Supernatants were analyzed for IL-17 production by ELISA and cells were subjected to quantitative RT-PCR for RORγt expression. Data are a representative of four similar experiments. D: Clinical EAE scores of animals treated daily from day 13 on (arrow) either with vehicle (CMC) or AM80. Displayed scores are representative of two experiments with $n = 6$.

rescence-activated cell sorting following re-stimulation with antigen did not differ between treated and control groups (Figure 3E). Collectively, these data suggest that rather acting as a systemic immunosuppressive agent, AM80 acts as an immunomodulary agent for antigen-specific T cell responses, primarily affecting Th17 cell function *in vivo*.

AM80 Treatment Ameliorates Ongoing Inflammatory Responses by Suppressing IL-17 Production from Differentiated Th17 Effector Cells

As we wished to evaluate the therapeutic potential of AM80 for MS treatment, we tested whether or not AM80 had an inhibitory effect on Th17 effector function in addition to its effect on Th17 differentiation. To this end, we stimulated memory cells in the presence of IL-23, a cytokine that promotes Th17 memory cell function and survival,[9] and in the presence of increasing doses of AM80 or ATRA. AM80 and ATRA suppressed IL-17 production by memory T cells responding to anti-CD3 mAb activation (Figure 4A). Intracellular cytokine staining also demonstrated that those retinoids inhibit IL-17 production by differentiated Th17 cells (AM80, Figure 4B; ATRA, data not shown). Furthermore, we also confirmed that IL-17 production by T cells differentiated *in vitro* by a combination of TGF-β and IL-6, was effectively inhibited in the presence of AM80 after restimulation with immobilized anti-CD3 mAb (data not shown). As Th17 cells are shown to have an unstable phenotype when differentiated *ex vivo*,[43] we examined how retinoids affect the function of Th17 cells that have stably differentiated *in vivo*. We stimulated CNS-infiltrating T cells isolated from mice at peak EAE, which consist of a high proportion of Th17 cells,[42] in the presence of AM80 or ATRA. Both RAR agonists successfully suppressed IL-17 production by those CNS infiltrating T cells concomitant with significant reduction of RORγt expression (Figure 4C). We further tested whether or not this effect was sufficient to modulate established EAE. Rescue treatment with AM80 starting after the onset of disease significantly suppressed the increase of disease scores that was observed in control vehicle-treated EAE mice (Figure 4D). The maximal disease score was reduced from 3.4 ± 0.39 in untreated EAE mice to 2.58 ± 0.47 in AM80-treated animals. Taken together, these data indicate the retinoid treatment is effective at inhibiting the function of activated Th17 cells.

Continuous AM80 Treatment Alters Cytokine Profile in the Chronic Phase of Disease

Our data have demonstrated that treatment with AM80 can both protect and rescue from EAE, associated with a suppression of pathogenic Th17 cell differentiation and function. However, when observed at later time points after EAE induction, the disease scores of both groups became alike (Figures 2A and 4D). Accordingly at such time points, there is no clear differences between treatment groups in terms of cellular infiltrates in CNS tissue as observed by histology (Figure 5A) or flow cytometry (Figure 5B). We then investigated the effector properties of CNS-infiltrating T cells derived from either vehicle-treated or AM80-treated animals with equivalent disease scores at day 25 after EAE induction. CNS-infiltrating T cells derived from AM80-treated animals contained strongly decreased levels of mRNA transcripts for RORγt, IL-23 receptor and IL-17 (Figure 5C). In addition, CNS-

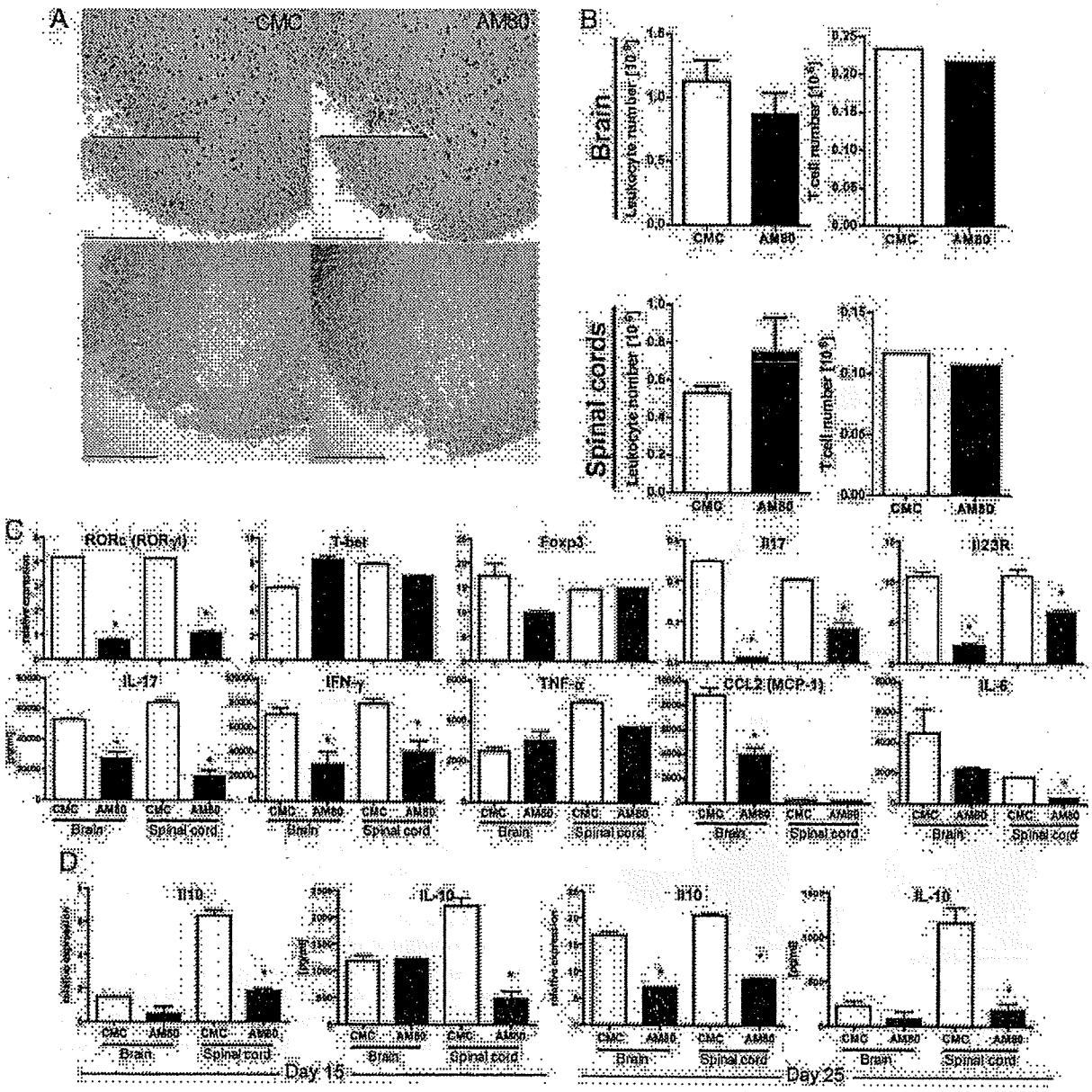

Figure 5. Continuous AM80 treatment is less effective on EAE suppression with a differentially modulated cytokine profile. **A:** The H&E-stained sections and the Luxol fast blue-stained sections in EAE mice treated with or without AM80 are shown. The control animal (score 2) and AM80-treated animal (score 2) at day 25 were subjected to histological examination. Scale bar = 200 μm. **B:** Total leukocyte numbers and isolated T cell numbers from spinal cords were evaluated in animals that had received CMC or AM80 at day 25 after immunization. The upper row of **C** depicts purified T cells from **B** that were subjected to quantitative RT-PCR. Error bars represent duplicated PCR of the same samples. In the lower row of **C**, CNS-infiltrating T cells were restimulated with immobilized anti-CD3 mAb and cytokine or chemokine production in culture supernatants were assessed by ELISA or CBA after 72 hours. Error bars represent measurements from duplicate wells (*$P < 0.001$). **D:** IL-10 production by stimulated CNS-infiltrating T cells derived from CMC-treated or AM80-treated animals were assessed by either quantitative RT-PCR or CBA. CNS-infiltrating T cells were isolated at day 15 or day 25 after EAE induction. **B** is a representative of four independent experiments; **A**, **C**, and **D** of two with six animals per group.

infiltrating T cells of AM80-treated animals produce significantly lower amounts of proinflammatory cytokines (IL-17, IFN-γ, and IL-6) and chemokines (CCL2) on restimulation *in vitro* (Figure 5C). Furthermore, although we have observed disease development until day 45, there were no further differences between treatment groups over this time period (data not shown). Collectively, these data suggest that inhibition of Th17 cell function alone is not sufficient to protect mice from later onset of the disease. Foxp3 expression in CNS infiltrating T by quantitative RT-PCR (Figure 5C) or intracellular Foxp3 staining (data not shown) showed no apparent differences between treatment groups, albeit a trend toward lower levels of Foxp3 expression in brain infiltrating T cells of AM80 treated animals could be observed. Next, we investigated several regulatory cytokines and found that levels of IL-10 were reduced in CNS-infiltrating T cell of animals that had received AM80 (Figure 5D). Interest-

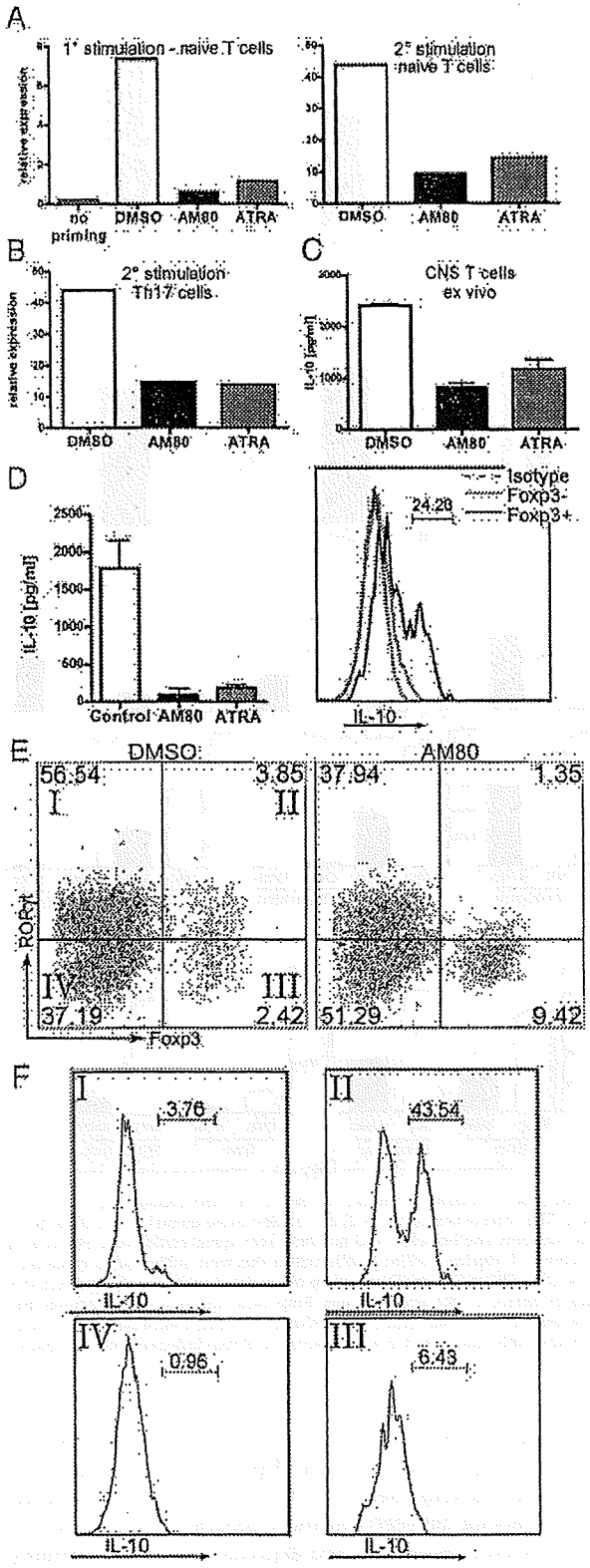

Figure 6. Continuous AM80 treatment impairs IL-10 production by T cells, which are identified as RORγt$^+$/FOXP3$^+$ population. A: Naïve T cells were cultured under neutral (no priming) or Th17-priming conditions in the presence of retinoids (100 nmol/L). After 96 hours, expression of IL-10 mRNA was assessed by quantitative RT-PCR. For further analysis, cells were rested for 48 hours and restimulated with anti-CD3 antibody for another 96 hours and examined for expression of IL-10 transcript. B: Naïve T cells stimulated under Th17 priming conditions without RAR agonists were then restimulated as described above. RAR agonists (100 nmol/L) were added during the secondary stimulation and assessed for their expression of IL-10 mRNA by quantitative RT-PCR. Results are representative of two independent experiments. C: CNS-infiltrating T cells were isolated from EAE animals (score 3) and restimulated with immobilized anti-CD3 mAb in the presence of AM80 or ATRA (100 nmol/L) for 72 hours. The amount of IL-10 in culture supernatants was examined by ELISA. D: Whole splenocytes were cultured for 96 hours with TGF-β and IL-6 and RAR agonists (10 nmol/L) and CBA analysis were performed for analyzing the levels of IL-10 production in the culture supernatants. Cells were then subjected to intracellular cytokine staining by fluorescence-activated cell sorting. The histogram gated on CD45^{high}TCR$^+$CD4$^+$ lymphocytes displays the comparative population of IL-10$^+$ cells by Foxp3$^+$ (black line) and Foxp3$^-$ (gray line) cell populations. Broken line represents the data stained with isotype control antibody. E: CD45^{high}TCR$^+$CD4$^+$ lymphocytes derived from D were analyzed for their expression of RORγt and Foxp3. Comparative dot plots were shown with or without AM80 treatment (10 nmol/L). Addition of AM80 decreased the number of RORγt$^+$ cells, including the RORγt$^+$FOXP3$^+$ cells. F: The levels of IL-10 producing cells from the quadrants labeled in E are shown.

ingly, we observed a more profound inhibitory effect of AM80 on IL-10 production by CNS-infiltrating T cells at the later time point after EAE induction (day 25) compared with those in earlier phases of the disease (day 15). Despite simultaneous suppression of IL-17 production, continuous AM80 treatment may deplete the host immune system of its intrinsic T cell production of IL-10, leading to a possible loss of the protective function of retinoids.

Continuous Treatment with RAR Agonists Abrogate IL-10 Production by T Cells, Which Are Identified To Be RORγtFoxp3 Double Positive

As continuous AM80 treatment suppressed the production of IL-10 by CNS-infiltrating T cells, we further investigated the effect of AM80 on the production of IL-10 *in vitro*. As shown in Figure 6A, we detected higher levels of IL-10 transcripts among effector T cells activated under Th17 priming conditions as compared with those primed under neutral conditions, supporting a previous study in which demonstrate increase IL-10 secretion in Th17 cultures on TGF-β and IL-6 signaling.[24] Addition of RAR agonists during the primary culture confers reduced expression of IL-10 transcripts, even when cells underwent a secondary stimulation in the absence of retinoids, pointing toward a stable phenotype. To assess the effect of retinoids on differentiated Th17 cells, T cells were primed under Th17 priming conditions without retinoids, but restimulation in the presence of retinoids decreased the expression of IL-10 (Figure 6B). In addition, *ex vivo* restimulation of CNS-infiltrating T cells in the presence of retinoids also reduced IL-10 expression (Figure 6C). Taken together, treatment with either AM80 or ATRA inhibits the production of IL-10 by Th17 cells, which have differentiated *in vitro* or *in vivo*.

Regulatory T cells have previously been indicated as main source of IL-10-producing T cell subsets.[44] In Figure 6D, we confirm that Foxp3$^+$ cells are the source of IL-10 in whole splenocyte stimulated in the presence of

TGF-β and IL-6. Interestingly, addition of retinoids expanded the number of Foxp3$^+$ cells (data not shown), but abolished their production of IL-10 (Figure 6D). Recently, a further subset of IL-10 producing regulatory T cells that express of RORγt and Foxp3 simultaneously have been highlighted.[25] Therefore, we hypothesized that such cells represent the major population of IL-10-secreting T cells in Th17 differentiation cultures and thus-it is these cells on which retinoids act to abolish IL-10 production. In fact, Foxp3$^+$ cells could be successfully divided into RORγt$^+$ and RORγt$^-$ populations (Figure 6E). Addition of AM80 to cultures reduced the proportion of RORγt$^+$ and RORγt$^+$Foxp3$^+$ double positive cells, but increased the proportion of Foxp3$^+$RORγt$^-$ cells (Figure 6E), at the same time as abolishing IL-10 production (Figure 6D). Intracellular IL-10 staining revealed that the RORγt$^+$Foxp3$^+$ double positive population was the predominant source of IL-10 (Figure 6F). Taken together, these data suggests that retinoid treatment reduces the production of IL-10 by inhibiting the effector function of a distinct RORγt$^+$Foxp3$^+$ population, which might serve as a regulatory T cell subset.

Discussion

ATRA, the physiologically active metabolite of vitamin A, inhibits the differentiation of Th17 cells and reciprocally promotes the generation of Treg cells *in vitro*.[27,29] In this study, we demonstrate for the first time that the synthetic RAR agonist AM80 inhibits the differentiation and effector function of Th17 cells more effectively than ATRA. Oral administration of AM80 attenuates antigen-specific Th17 cell differentiation, thus such treatment is able to reduce disease in the acute phase of EAE.

ATRA suppresses Th17 differentiation and effector function via RARα signaling,[27,28] but ATRA can also bind to RARβ and RARγ, which can form a variety of homo- and heterodimers with three RXR receptors.[26,34] Non-selective receptor binding is thought to be a major cause of the side effects associated with the administration of ATRA and other pan-RAR agonists in the clinic. AM80 is a synthetic RAR agonist that has high affinity to the RARα/β and is currently available as medication for human diseases such as APL and psoriasis.[35–37,45] In addition to greater specificity for RARα, AM80 offers several other advantages over ATRA as a therapeutic agent: it has a lower toxicity, higher stability, fewer potential side effects, and a superior bioavailability. Also, we demonstrate that a lower dose of AM80 is required to inhibit IL-17 production by T cells than similar treatment with ATRA. Interestingly, at very low doses, AM80 treatment reduced IL-17 production dramatically, while RORγt expression was only slightly reduced. Although RORγt expression is a pre-requisite for Th17 development,[17] recent studies have demonstrated that Foxp3 can physically interact with RORγt inhibiting IL-17 production when both are co-expressed.[25,46,47] It is conceivable that the disconnection between RORγt expression and IL-17 secretion following low dose AM80 treatment we observed may result from concomitant Foxp3 up-regulation. However, at higher doses of AM80, the inhibition of IL-17 production was associated with a reduction in RORγt expression; thus there may be multiple mechanisms of action in operation.

A recent study demonstrated that intraperitoneal injection of high doses of ATRA protected animals from EAE induction and that this protection was associated with reduced IL-17 and IFN-γ production.[30] However, such treatment was also found to reduce proliferative T cells responses following antigen restimulation *ex vivo*,[30] suggesting that, in this study, ATRA may ameliorate EAE by generating systemic immune-suppression. We have observed similar immunosuppression by ATRA and other retinoids at a high dose (data not shown). In our study, we treated with AM80 at a dose 5–10 times lower than the dose that ATRA has been previously tested at and we were able to administer the retinoid orally. Our treatment regimen also suppressed Th17 cell differentiation and IL-17 production, but antigen-specific T cell proliferation was not altered. Thus, we were able to target pathogenic Th17 cells specifically, without inducing general immunosuppression.

We and others have found that there is no induction of regulatory T cells when treating inflammatory diseases with RAR agonists,[29,30] and it has been speculated that this may be due to a lack of TGF-β *in vivo*.[29] An alternative hypothesis is that Treg generation is inhibited by the strong induction of inflammatory cytokines, including IL-6, TNF- α, and IL-1.[30] Thus, under both of these suggestions, it is likely that AM80 suppresses EAE by inhibiting the generation and activity of Th17 cells.

AM80 treatment inhibited acute EAE in mice, but continuous administration of AM80 did not suppress chronic inflammation. Interestingly, T cells isolated from the CNS tissue of AM80-treated mice during the chronic phase of the disease continued to express only low levels of RORγt, IL-23 receptor, and IL-17. This was in marked contrast to vehicle-treated control mice, which, despite analogous clinical disease scores, had CNS-infiltrating T cells that expressed high levels of Th17-related factors. Thus, we suggest that inhibition of Th17 cell function alone is not sufficient to protect mice from chronic CNS inflammation.

In fact, we found that treatment with RAR-agonists suppressed T cell production of IL-10 at late stage disease. Recently, a unique T cell subset that co-expresses RORγt and Foxp3, and predominantly secretes regulatory IL-10 has been identified *in vivo*.[23] Intriguingly, we show that RAR agonists not only suppress pathogenic Th17 cells, but also suppress IL-10 production by these RORγt+Foxp3+ T cells. Korn *et al* propose a model of sequential infiltration by different subsets of differentiated CD4$^+$ T cells during organ-specific autoimmunities such as EAE. In this model, Th17 cells mediate the acute phase of disease, while Th1 cells are more prominent in the chronic phase, and at much later phase of disease, there is a moderate up-regulation of IL-10 production.[48,49] In addition, previous studies indicate that IL-10 is a key cytokine for the suppression of T cell-mediated autoimmune inflammation in the CNS.[23,49] Therefore, the residual expression of IL-17 or IFN-γ at later time points

with continuous AM80 treatment may cause moderate progression of EAE development under the condition with reduced IL-10 production *in vivo*. We have little information about how those two subsets of IL-17-producing inflammatory RORγt+ T cells and IL-10-producing immunoregulatory RORγt+Foxp3+ T cells developed during immunological responses. Although we demonstrate here that the suppressive effect of AM80 on EAE is most likely through modulation of T cell production of both IL-17 and IL-10 *in vivo*, we don't exclude the possible involvement other factors, as EAE/MS are complex diseases that do not depend only on a IL-17 versus IL-10 dichotomy. For example, ongoing neurodegeneration[50] may also contribute significantly to the observed phenotype especially at the later phase of disease. As Th1 cells also play a significant role in the induction or EAE/MS, we should also point out that retinoic acid has been previously shown to exert direct effects on T cells, suppressing Th1 development and enhancing Th2 development via retinoic acid receptors.[51] This indicates that AM80 has multiple beneficial effects for disease protection through oral administration. The immunomodulatory effect of RAR agonists on those Th17-like regulatory T cells in inflammatory autoimmune diseases is to be considered if conducting *in vivo* attenuation of Th17 cells for treatment of autoimmune diseases with retinoids. At this point in time, we advocate that the use of AM80 on inflammatory autoimmune diseases should target the acute phases of Th17-mediated pathogenesis.

In addition, we found that T cells that had infiltrated the spinal cord produced much lower amounts of CCL2 (MCP-1) as compared with brain-infiltrating T cells. CCL2 plays a crucial role in the progression of EAE[52,53] and previously we have shown that human Th17 cells express the corresponding receptor, CCR2.[54] Our data showing reductions in CCL2 in the spinal cord may represent previously reported differences in inflammatory cell populations at different CNS sites.[55]

In summary, we demonstrate that oral treatment with AM80 effectively inhibits IL-17 production without generating systemic immunosuppression. In addition, AM80 treatment protected mice from the development of acute EAE and rescued mice with established EAE from acute autoimmune inflammation. Collectively, these data advocate AM80 as a potent therapeutic agent against acute Th17-mediated autoimmune diseases including MS.

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Suppression of Experimental Autoimmune Encephalomyelitis by Ghrelin[1]

Michael-Mark Theil,*† Sachiko Miyake,[2]* Miho Mizuno,* Chiharu Tomi,* J. Ludovic Croxford,* Hiroshi Hosoda,‡ Julia Theil,*† Stephan von Hörsten,†§ Hiroaki Yokote,* Asako Chiba,* Youwei Lin,* Shinji Oki,* Takashi Akamizu,¶ Kenji Kangawa,¶ and Takashi Yamamura[2]*

Ghrelin is a recently identified gastric hormone that displays strong growth hormone-releasing activity mediated by the growth hormone secretagogue receptor. While this unique endogenous peptide participates in the regulation of energy homeostasis, increases food intake, and decreases energy expenditure, its ability to inhibit the production of proinflammatory cytokines in vitro indicates its role in the regulation of inflammatory process in vivo. Here we examine the effect of exogenous ghrelin on the development of experimental autoimmune encephalomyelitis (EAE), a representative model of multiple sclerosis. In the C57BL/6 mouse model of EAE induced by sensitization to myelin oligodendrocyte glycoprotein 35–55 peptide, we found that alternate-day s.c. injections of ghrelin (5 μg/kg/day) from day 1 to 35 significantly reduced the clinical severity of EAE. The suppression of EAE was accompanied by reduced mRNA levels of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 in the spinal cord cellular infiltrates and microglia from ghrelin-treated mice at the peak of disease, suggesting the role of ghrelin as an antiinflammatory hormone. Consistently, ghrelin significantly suppressed the production of proinflammatory cytokines in LPS-stimulated microglia in vitro. These results shed light on the new role of ghrelin in the regulation of inflammation with possible implications for management of human diseases. *The Journal of Immunology,* 2009, 183: 2859–2866.

Small synthetic compounds, referred to as growth hormone (GH)[3] secretagogues (GHS), have been known to stimulate GH release, working through a G protein-coupled receptor called GHS receptor (GHS-R) (1–3). It is now established that a new endogenous peptide, ghrelin, discovered in rat gastric extracts, is an endogenous ligand for GHS-R and is involved in the regulation of GH release. Ghrelin is a 28-aa polypeptide with an essential *n*-octanoyl modification on serine at position 3 (4). Although ghrelin is predominantly secreted from mucosal endocrine cells of stomach, it is widely distributed in various organs, including lymphoid tissues (5, 6). Furthermore, it is measurable in the systemic circulation, indicating its hormonal nature (7).

Ghrelin does not only stimulate GH release, but it also increases food intake, regulates energy homeostasis, and decreases energy expenditure by lowering the catabolism of fat (4, 8, 9). Because of its orexigenic and adipogenic character, ghrelin may be potentially useful for the treatment of anorexia and cachexia (10, 11). Although the precise mechanisms remain to be clarified, the orexigenic activities of ghrelin may be mediated by another feeding regulatory hormone neuropeptide Y (NPY) via stimulation of Y1 and Y5 receptors (12). Furthermore, the antagonistic effect of ghrelin on leptin-induced decrease of food intake seems to be mediated by ghrelin-induced release of NPY and subsequent stimulation of the Y1 receptor (13).

Ghrelin has been shown to exhibit antiinflammatory functions against T cells and macrophages in vitro (14–16). The potential activity of ghrelin as antiinflammatory reagent in vivo was shown in several animal models, including bowel disease (17), arthritis (16, 18), sepsis, and endotoxemia (16, 19, 20). Here we report that s.c. injections of ghrelin could significantly attenuate the clinical severity of the representative model of experimental autoimmune encephalomyelitis (EAE) induced in C57BL/6 (B6) mice by sensitization against myelin oligodendrocyte glycoprotein $(MOG)_{35-55}$ peptide. Furthermore, we demonstrate that in vivo treatment with ghrelin significantly suppressed the mRNA levels of the proinflammatory cytokines TNF-α, IL-1β, and IL-6 in microglia and infiltrating T cells derived from the spinal cords of ghrelin-treated mice. Finally, we confirm that LPS-stimulated microglia and monocytes produced lower amounts of proinflammatory cytokines when they were pretreated with ghrelin in vitro. In conclusion, the present study indicates the potential use of ghrelin as an antiinflammatory drug to control human CNS pathology.

Materials and Methods

Mice and reagents

We used female B6 mice (CLEA Japan) between 6 and 10 wk of age in specific pathogen-free conditions. Animal care and use were in accordance

*Department of Immunology, National Institute of Neuroscience, National Centre of Neurology and Psychiatry, Tokyo, Japan; †Department of Functional and Applied Anatomy, Hannover Medical School, Hannover, Germany; ‡Department of Biochemistry, National Cardiovascular Center Research Institute, Fujishirodai, Suita, Osaka, Japan; §Experimental Therapy, Franz-Penzoldt-Center, Friedrich-Alexander-University Erlangen-Nürnberg, Erlangen, Germany; and ¶Ghrelin Research Project, Translational Research Center, Kyoto University Faculty of Medicine, Kyoto, Japan

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[2] Address correspondence and reprint requests to Dr. Sachiko Miyake or Dr. Takashi Yamamura, Department of Immunology, National Institute of Neuroscience, National Centre of Neurology and Psychiatry, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187-8502. E-mail address: miyake@ncnp.go.jp or yamamura@ncnp.go.jp

[3] Abbreviations used in this paper: GH, growth hormone; EAE, experimental autoimmune encephalomyelitis; GHS, growth hormone secretagogue; GHS-R, growth hormone secretagogue receptor; LN, lymph node; MOG, myelin oligodendrocyte glycoprotein; NPY, neuropeptide Y.

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Table I. *Amino acid sequence of mouse ghrelin and des-acyl ghrelin*

Peptide	Amino Acid Sequence[a]	Ser^3 acylation	Reference
Ghrelin	GS<u>S</u>FLSPEHQKAQQRKESKKPPAKLQPR	*n*-Octanoicacid	(4)
Des-acyl ghrelin	GS<u>S</u>FLSPEHQKAQQRKESKKPPAKLQPR		(7)

[a] The underlined letter S represents the third serine (Ser^3).

with institutional guidelines. Animal experiments were approved by our institutional review committee. Rat MOG_{35-55} (amino acid sequence MEVGWYRSPFSRVVHLYRNGK) was synthesized at Toray Research Center (Tokyo, Japan). Ghrelin and des-acyl ghrelin (Table I) were synthesized as previously described (4, 7).

Immunization and clinical assessment of EAE

We immunized mice ($n = 5$–15 per group) s.c. in the tail base with 100 μg of MOG_{35-55}-peptide dissolved in 0.1 ml of PBS and 0.1 ml of CFA containing 1 mg of *M. tuberculosis* H37Ra (Difco). Shortly after immunization and 48 h later, the mice were injected i.p. with 200 ng of pertussis toxin (List Biological Laboratories). Clinical scores of EAE were daily assigned as follows: 0, normal; 1, weakness of the tail and/or paralysis of the distal half of the tail; 2, loss of tail tonicity; 3, partial hind limb paralysis; 4, complete hind limb paralysis; 5, forelimb paralysis or moribund; 6, death. The cumulative scores were calculated for individual mice by summing up the daily scores.

Administration of ghrelin and des-acyl ghrelin

For EAE treatment, we s.c. injected ghrelin and des-acyl ghrelin diluted in 0.9% saline. In the first series of experiments, mice were injected with ghrelin or des-acyl ghrelin at doses of 0.5, 5, or 50 μg/kg every other day for 35 days. Sham-treated animals were injected with 0.9% saline (standard protocol). In the next experiment, we injected the mice with 5 μg/kg ghrelin every day from day 1 to 10 (induction phase treatment) or from day 11 to 20 (effector phase treatment) and in-between with 0.9% saline. The controls were injected every day from day 1 to 20 with 0.9% saline (alternative protocol).

Assessment of histological EAE

To evaluate the histological manifestations of EAE, we treated mice with 5 μg/kg ghrelin or 0.9% saline following the standard protocol and sacrificed them on day 17 postimmunization. The spinal cords were removed and fixed in buffered formalin. They were embedded in paraffin, sectioned, and stained with H&E and Luxol fast blue for histopathological analysis.

Flow cytometry and isolation of mononuclear cells from the CNS

B6 mice were challenged for EAE, treated following the standard protocol with 5 μg/kg ghrelin or 0.9% saline and sacrificed on day 17 postimmunization. We removed spleen, lymph nodes (LN), and thymus as well as spinal cord from the ghrelin- and saline-treated mice for flow cytometer analysis. Single-cell suspensions were prepared according to standard methods. The spinal cord cell suspensions were centrifuged at 200 × *g* for 10 min and resuspended in 4 ml of 70% isotonic Percoll (Amersham Biosciences)/PBS and overlaid by equal volumes of 37% and 30% isotonic Percoll. The gradient was centrifuged at 500 × *g* for 15 min and the mononuclear cells were harvested from the 37%–70% interface, washed, and counted. The cells were stained for 5 min with anti-FcRγ III/II mAb (BD Pharmingen), washed, and labeled with the following mAbs for surface phenotype analysis: FITC-CD4 mAb, FITC-CD19 mAb, PE-CD8a mAb, PE-NK1.1 mAb, PE-CD25 mAb, allophycocyanin-FOXP3, and PerCP-Cy5.5-CD3e mAb (BD Pharmingen) and FITC-F4/80 mAb (Dainihon Seiyaku). The cytofluorometric analysis was performed using a FACSCalibur operated by CellQuest software (BD Biosciences).

Cytokine and cell proliferation assay

MOG_{35-55}-immunized B6 mice were treated s.c. with 5 μg/kg/day of ghrelin or 0.9% saline every day from day 1 to 10. The LN cells were collected on day 11 after immunization and suspended in our standard lymphocyte culture medium (RPMI 1640 supplemented with 5×10^{-5} M 2-ME, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin) added with 1% syngeneic mouse serum. The cells were cultured in 96-well round-bottom plates at 1×10^6/well for 72 h in the presence of 100 μg/ml MOG_{35-55}. Levels of IFN-γ, IL-17, and IL-4 in the supernatant were determined by using a sandwich ELISA. Proliferative responses were measured using a Beta-1205 counter (Pharmacia) to detect the incorporation of [^{3}H]thymidine (1 μCi/well) for the final 16 h of culture.

Evaluation of encephalitogenic T cell induction in B6 mice treated with ghrelin

To evaluate whether in vivo ghrelin treatment may affect the induction of encephalitogenic T cells after immunization with MOG_{35-55}, we evaluated the ability of the lymphoid cells from ghrelin- or saline-treated mice to passively transfer EAE into naive recipients. Donor B6 mice were immunized with MOG_{35-55} and treated every day from day 1 to 10 with 5 μg/kg/day of ghrelin or 0.9% saline. We removed spleens and LN from the donor mice on day 11 and prepared lymphoid cell suspensions. The lymphoid cells were stimulated with MOG_{35-55} (33 μg/ml) in the standard medium added with FCS (10%) for 96 h and then we isolated the $CD4^+$ T cells for cell transfer by depletion of $CD8^+$, $CD19^+$, and $NK1.1^+$ cells. In brief, the MOG_{35-55}-stimulated total lymphoid cells were labeled with PE-CD8a mAb, PE-NK1.1 mAb, and PE-CD19 mAb (BD Pharmingen) for 30 min, washed, and incubated with anti-PE microbeads (Miltenyi Biotec) for 15 min. Using autoMACS (Miltenyi Biotec), we isolated $CD4^+$ T cells ($CD8^-$, $CD19^-$, and $NK1.1^-$ fraction) as a pass-through and suspended the cells in PBS. We injected 1.0×10^7 of the cells into the peritoneal cavity of syngeneic recipient mice that had been X-irradiated (550 rad) shortly before. We also injected 200 ng of pertussis toxin i.p. on the same day and 48 h later.

Reverse transcription and real-time PCR

To analyze the mechanism of ghrelin effects in vivo, we extracted total RNA from spinal cord, spleen, thymus, and LN samples using the RNeasy Mini Kit (Qiagen). The RNA was subjected to reverse transcription with the Advantage RT-for-PCR kit (BD Biosciences). Real-time PCR was conducted in the LightCycler quantitative PCR system (Roche Molecular Biochemicals) by using the LightCycler-FastStart DNS Master SYBR Green I kit (Roche Molecular Biochemicals). We followed the manufacturer's specification using 4 mM $MgCl_2$ and 1 pM primers. The primers used are as follows: TNF-α, CTGTGAAGGGAATGGGTGTT (sense) and GGT CACTGTCCCAGCATCTT (antisense); IL-1β, TGAAATGCCACCTTT TGACA (sense) and GTAGCTGCCACAGCTTCTCC (antisense); IL-6, TTCCATCCAGTTGCCTT–CTT (sense) and CAGAATTGCCATTGC ACAAC (antisense); TGF-β, TGCGCTTGCAGA–GATTAAAA (sense) and GCTGAATCGAAAGCCCTGTA (antisense); and HPRT, GTTGGA–TACAGGCCAGACTTTGTTG (sense) and GAGGGTAGGCTGGCCT ATAGGCT (antisense). Values are presented as the relative amount of transcript of each sample normalized to the housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT).

In vitro effect of ghrelin on RAW 264.7 monocytes treated with LPS

To examine the effect of ghrelin on monocytes, RAW 264.7 monocytes (American Type Culture Collection) were suspended in the standard culture medium supplemented with 10% FCS and cultured in 96-well flat bottom plates at 1×10^5/well overnight. Various concentrations of ghrelin (10^{-6} M, 10^{-8} M, 10^{-10} M) were added to the culture and 1 h later the cells were stimulated with LPS (Sigma-Aldrich) at various doses (0.1, 1, 10 μg/ml). After 2 h of incubation at 37°C, supernatants were collected and the levels of TNF-α and IL-6 were detected by using a sandwich ELISA.

Isolation of microglial cells from the CNS

The spinal cords were incubated with 35 mg/ml Liberase Blendzyme 3 (Roche Molecular Biochemicals) and 0.1 mg/ml DNaseI (Roche Molecular Biochemicals) in RPMI 1640 medium at 37°C for 30 min. Mononuclear cells were isolated on 30%–80% discontinuous Percoll gradients and were stained with FITC-CD11b mAb, PE-CD45 mAb, and allophycocyanin-CD3 mAb (BD Pharmingen). $CD11b^{high}CD45^{high}$ macrophage cells, $CD11b^{int}CD45^{int}$ microglial cells, and $CD3^+$ T cells were isolated using

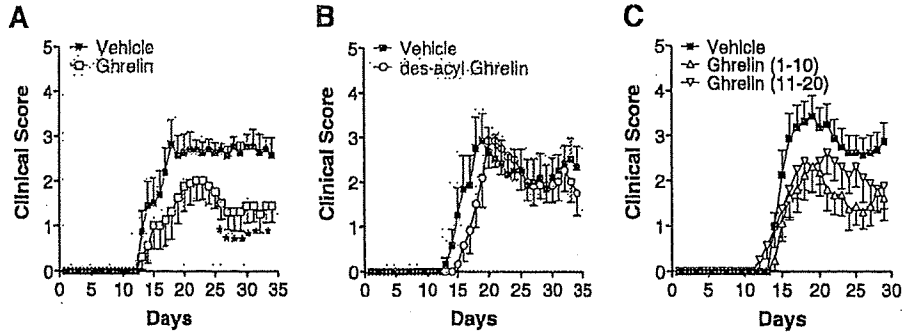

FIGURE 1. Effect of ghrelin on actively induced EAE. EAE was induced in female B6 mice (n = 8 in each group of the three experiments) by immunization with MOG_{35-55}. *A*, The mice were treated every other day starting at the day of immunization with 5 μg/kg ghrelin, while controls were administrated 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.

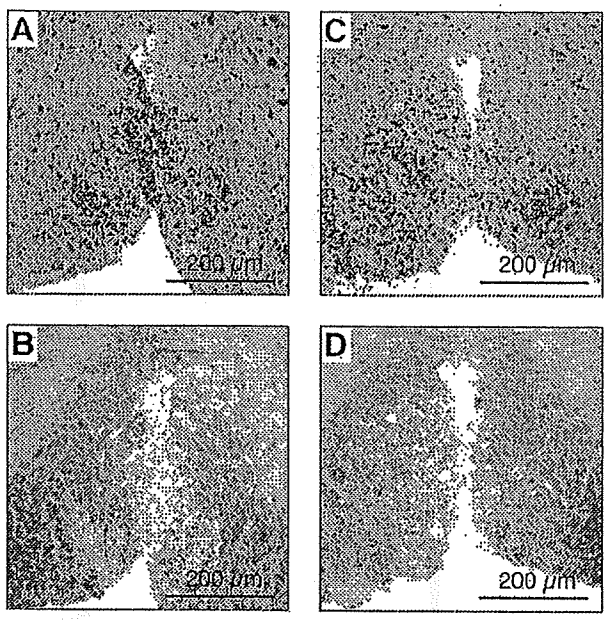

FIGURE 2. Histopathological assessment of the spinal cord of EAE mice. Spinal cords from EAE mice (n = 5/group) were removed on day 17 postimmunization as described in *Material and Methods*. The spinal cord sections from sham- (*A* and *B*) and ghrelin-treated (*C* and *D*) mice were stained in with 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

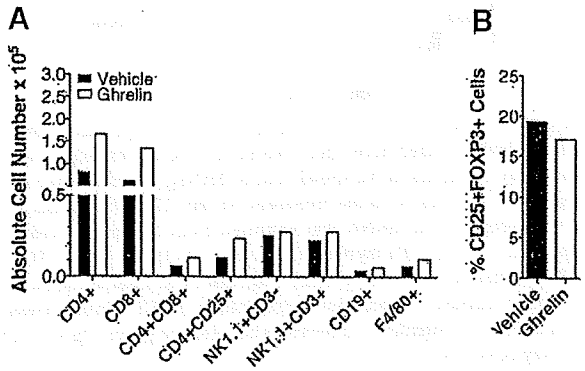

FIGURE 3. Quantification of spinal cord cellular infiltrates by flow cytometry. *A*, The cells were isolated from spinal cords of ghrelin- and sham-treated mice on day 17 postimmunization and subjected to flow cytometer analysis as described in *Materials and Methods*. Data are representative of two 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

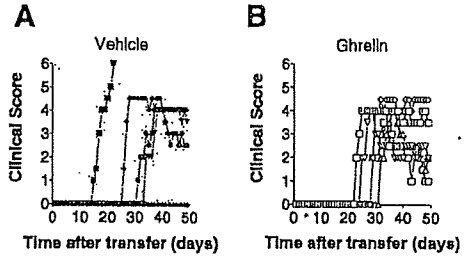

FIGURE 4. Effects of ghrelin treatment on the induction of encephalitogenic T cells. MOG_{35-55}-sensitized lymphoid cells were derived from MOG_{35-55}-immunized and (*A*) saline- or (*B*) ghrelin-treated mice (n = 15/group). The cells were stimulated with MOG_{35-55} and $CD4^+$ T cells were separated 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

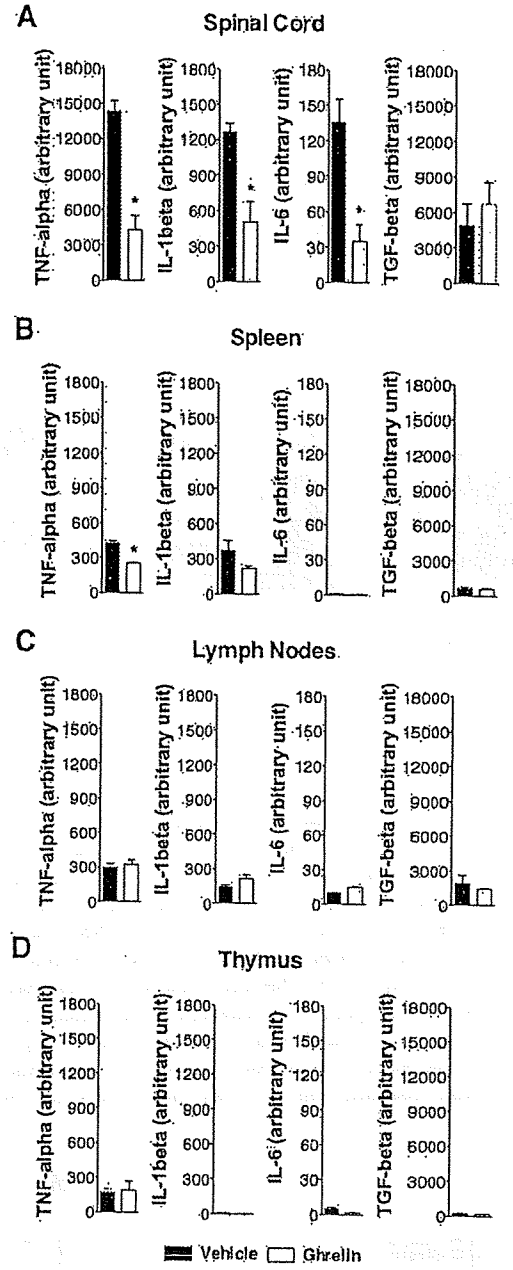

FIGURE 5. Proinflammatory cytokine mRNA expression during EAE in ghrelin-treated mice. Quantitative mRNA expression of proinflammatory cytokines in the spinal cord of MOG_{35-55}-immunized mice subjected to ghrelin or saline treatment on day 17 postimmunization (n = 5/group). Total mRNA was extracted 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

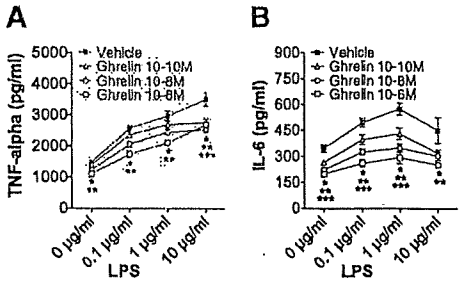

FIGURE 6. Effect of ghrelin on the proinflammatory cytokine production of LPS-stimulated monocytes. The monocytes were treated with various concentrations of ghrelin (10^{-6} M, 10^{-8} M, 10^{-10} M) 1 h before stimulation with 0.1, 1.0, and 10 µg/ml LPS. The (*A*) TNF-α and (*B*) IL-6 production was 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.

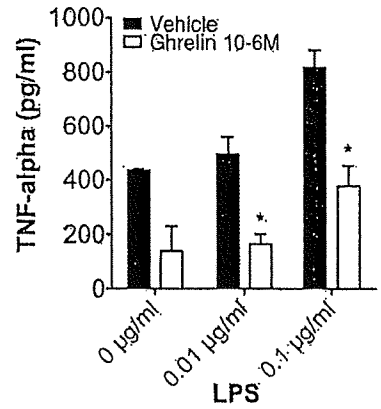

FIGURE 8. Effect of ghrelin on the proinflammatory cytokine production of LPS-stimulated microglia. The microglia cells were treated with ghrelin (10^{-6} M) overnight and later stimulated with 0.01 and 0.1 µg/ml LPS. Five hours after stimulation, the TNF-α production was measured using ELISA. Data represent 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

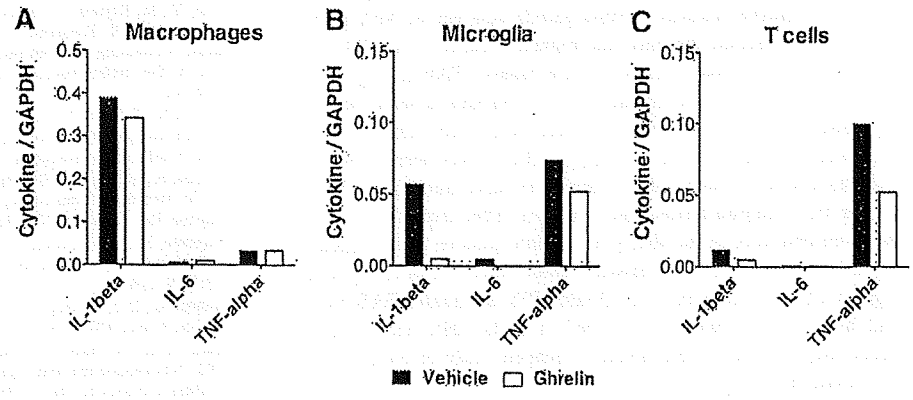

FIGURE 7. Effect of ghrelin on proinflammatory cytokine mRNA expression in infiltrating cells and microglia. Total mRNA was extracted from (*A*) macrophages, (*B*) microglia, and (*C*) T cells obtained on day 20 postimmunization from the spinal cords of MOG_{35-55}-immunized mice treated with ghrelin 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

Mayumi Fujita,* Takao Otsuka,*† Miho Mizuno,* Chiharu Tomi,* Takashi Yamamura,* and Sachiko Miyake*

From the Department of Immunology, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo; and the Third Department of Internal Medicine,† Tokyo Medical University, Tokyo, Japan*

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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M.F. and T.O. contributed equally to this work.

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Address reprint requests to Sachiko Miyake, Department of Immunology, National Institute of Neuroscience, NCNP, Kodaira, Tokyo 187-8502, Japan. E-mail: miyake@ncnp.go.jp.

(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

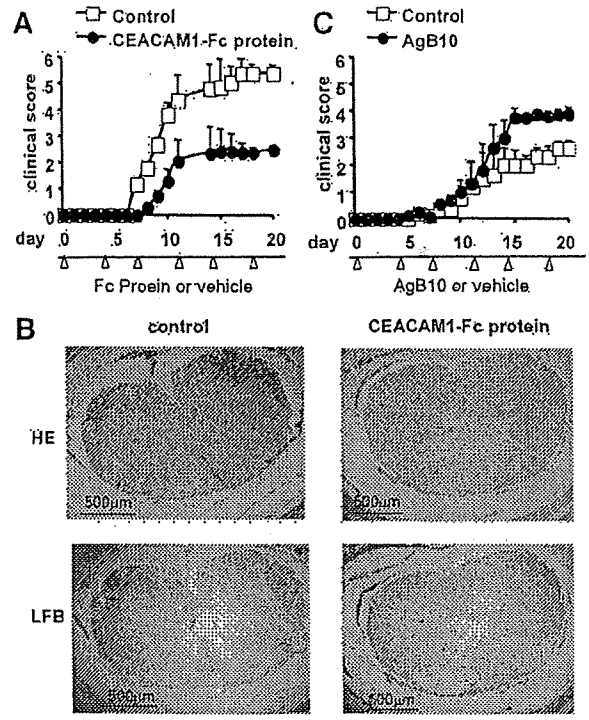

Figure 1. Effect of CEACAM1-Fc fusion protein or CEACAM1-specific antibody on MOG-induced EAE. EAE was induced in B6 mice by immunization with MOG_{35-55}. CEACAM1-Fc fusion protein (A) or a mAb for CEACAM1, AgB10 (C) was given twice per week starting from the day of immunization. **Arrowheads** indicate 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.

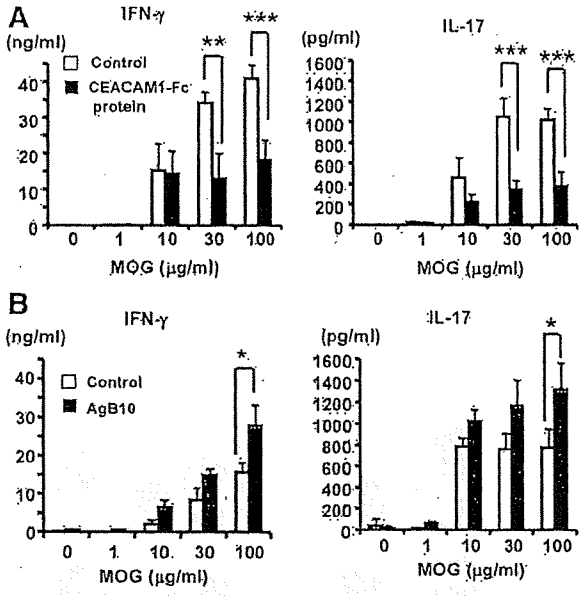

Figure 2. MOG_{35-55}-specific T cell responses in mice treated with CEACAM1-Fc fusion protein or with AgB10. B6 mice were treated with CEACAM1-Fc fusion protein (A) or AgB10 (B) twice per week from the day of immunization with MOG_{35-55}. Eleven days after the immunization, draining lymph node cells 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.

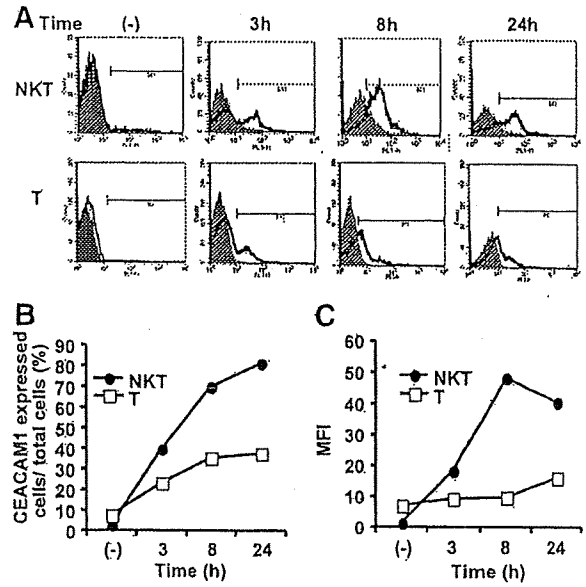

Figure 3. Expression of CEACAM1 on iNKT and T cells, after activation with ConA and anti-CD3 antibody. The histograms show the log fluorescence intensity of CEACAM1 on the surface of iNKT and T cells at the indicated time points after the activation with a combined treatment of ConA and plate bounded 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

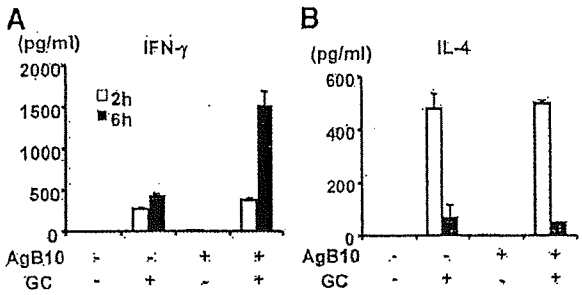

Figure 4. Effect of AgB10 on serum cytokine levels after *in vivo* injection with α-galactosylceramide (α-GC). B6 mice were treated with either AgB10 or control antibody. Four days after the treatment, serum was collected at 2 or 6 hours after intravenous injection of α-GC. Serum levels of IFN-γ and 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.

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).

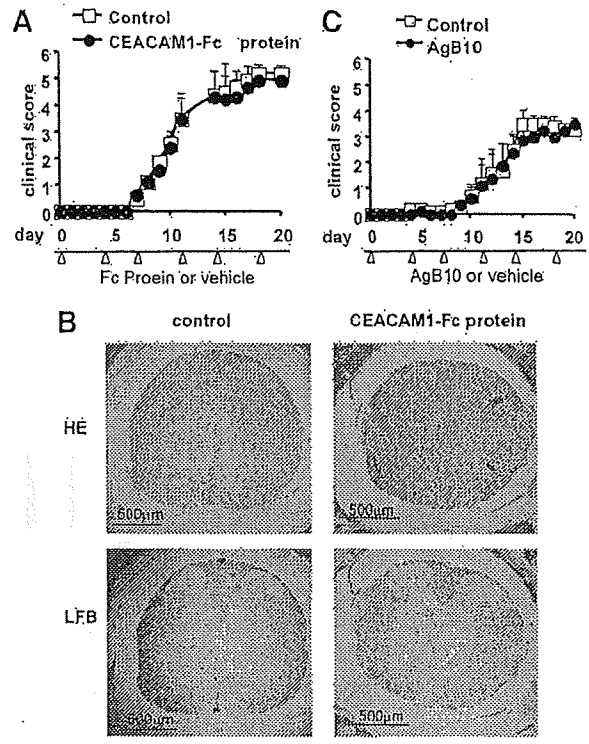

Figure 5. Effect of CEACAM1-Fc fusion protein or CEACAM1-specific antibody on EAE induced in Jα18$^{-/-}$ mice. EAE was induced in *Jα18*$^{-/-}$ mice by immunization with MOG_{35-55}. CEACAM1-Fc fusion protein (A) or AgB10 (C) was given twice per week starting from the day of immunization. Arrowheads 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.

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).

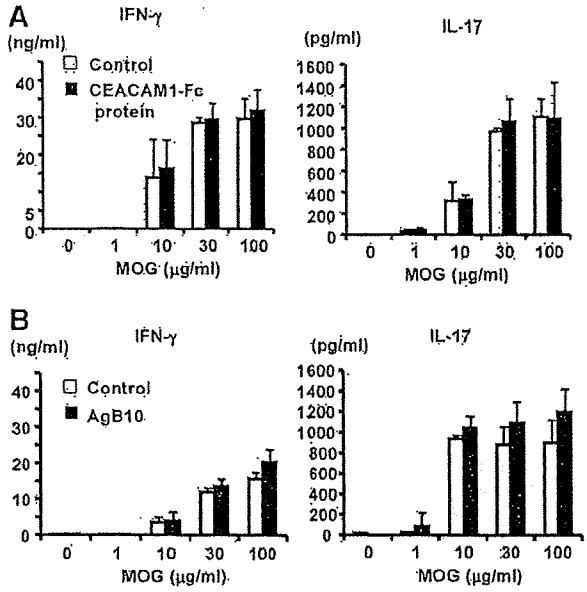

Figure 6. MOG_{35-55}-specific T cell responses in $J\alpha18^{-/-}$ mice treated with CEACAM1-Fc fusion protein or with AgB10. $J\alpha18^{-/-}$ mice were treated with CEACAM1-Fc fusion protein (A) or AgB10 (B) twice per week from the day of immunization with MOG_{35-55}. Eleven days after the immunization, 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.

Acknowledgments

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