continue to increase over 7–13 days postadministration and remain constant for up to 23 days [87]. FTY720 studies in animals have demonstrated an immunosuppressant effect of sequestering T and B cells in peripheral lymphoid tissue [88]. In addition, EAE studies show that FTY720 can prevent [89,90] or reverse established inflammatory CNS disease [90,91] by limiting T-cell infiltration of the CNS and concomitant reduction of CNS proinflammatory Th1-cytokine mRNA transcripts. Furthermore, FTY720 may play a role in the structural restoration of the CNS parenchyma by direct modulation of CNS resident glial cells [92].

These animal studies, therefore, provide a rationale for use in MS clinical trials. In a proof-of-concept clinical trial, 281 patients were randomly assigned treatment with FTY720 (1.25 or 5 mg orally) daily or placebo for 6 months [93]. The primary end point was the number of gadolinium-enhancing lesions per month over the 6-month duration. Patients receiving FTY720 treatment experienced fewer lesions than the placebo group (1.25 mg: one lesion; $p < 0.001$, 5 mg: three lesions; $p < 0.006$, placebo: five lesions). In addition, the annualized relapse rate was reduced in the FTY720-treated group compared with placebo: 0.35 for 1.25 mg ($p = 0.009$) and 0.36 for 5 mg FTY720 ($p = 0.01$) versus 0.77 for placebo. After 6 months, some patients on placebo were further randomized to receive FTY720 treatment. Lesion numbers and relapse rates in these patients also decreased compared with placebo. FTY720 was well tolerated with reported adverse events, including headache, diarrhea, nausea, dyspnea (shortness of breath) and nasopharyngitis [93]. An increase in alanine aminotransferase was also observed in some patients. A recent study to determine the mode of action of FTY720 in MS clinical trials demonstrated that it inihibits the S1P/S1P1-dependent T-cell trafficking from peripheral lymph nodes [94]. Accordingly, peripheral blood counts from treated MS patients demonstrated a significant reduction of $CD4^+$ and $CD8^+$ T cells, 80 and 60%, respectively, compared with samples from patients receiving IFN-β therapy [94]. The study observed a differential effect upon T-cell subsets. Both naive and central memory T cells, which both express the chemokine receptor CCR7, were selectively inhibited whereas peripheral effector T cells (CCR7 negative) were unaffected [94].

FTY720 appears to be a promising candidate for future MS therapy and the ability to deliver FTY720 orally to patients is of great benefit as patients are more likely to continue treatment in the absence of injection-site adverse events. Although the main mechanism of action appears to be inhibition of T-cell trafficking to the CNS by sequestration of cells to peripheral lymphoid organs, the abundant expression of S1P receptors throughout the CNS means that FTY720 may act both as an immunomodulator and also as a neuroprotective agent, although its effects on neuroprotection remain to be elucidated.

Teriflunomide

Teriflunomide is a metabolite of leflunomide, a potent disease-modifying antirheumatic drug. It is thought to have immunomodulatory properties that result from its ability to inhibit the transcription factor NF-κB and pyrimidine *de novo* synthesis by blocking the dihydoorotate dehydrogenase enzyme. However, teriflunomide was shown to inhibit both T-cell receptor (TCR)–CD3-mediated calcium mobilization and the formation of an immunological synapse between T cells and antigen-presenting cells (APCs) independent of altered pyrimidine synthesis [95]. In addition, other critical T-cell signaling molecules, such as MAPK and NF-κB, were unaffected. Using adoptive transfer models of EAE, it was demonstrated that 7-day treatment of teriflunomide could suppress disease in rats [96]. Myelin-specific T cell antigens activated *in vitro* were induced to secrete less IFN-γ but increased levels of IL-10. Furthermore, when these cells were transferred to naive mice, they transferred a less severe form of disease, probably as a result of suppressed chemotaxis [96].

A randomized, double-blind, placebo-controlled Phase II clinical trial to study the efficacy and safety of teriflunomide in MS patients used 179 patients of whom 157 had RR-MS and 22 had SP-MS [97]. Patients received either placebo, or teriflunomide doses of 7 or 14 mg/day orally for 36 weeks. MRI scans were performed every 6 weeks. The primary end point was the number of unique active lesions per scan and secondary end points included MRI-measured disease burden, relapse frequency and disability progression. The study found that the median number of new and active lesions in the drug-treated groups was significantly lower (7 mg/day: 0.2, $p < 0.03$; 14 mg/day: 0.3, $p < 0.01$) compared with placebo (0.5). In addition, fewer people receiving the higher dose of teriflunomide demonstrated disability increase compared with placebo. No effect on relapse rate was observed. Teriflunomide was well tolerated with headaches and upper respiratory tract infections the most

common adverse events. A Phase III trial called Study of Teriflunomide in Reducing the Frequency of Relapses and Accumulation of Disability in Patients With Multiple Sclerosis (TEMSO) to assess whether teriflunomide is able to reduce the frequency of relapses and the accumulation of disability has been completed and published results are awaited. Further studies of efficacy and safety of teriflunomide in conjunction with IFN-β are being conducted in Phase II trials.

Laquinimod

Laquinimod is an orally bioavailable quinoline-3-carboxamide that has been shown to inhibit the disease course in animal models of MS. Oral administration of laquinimod was effective at both preventing and reversing established EAE, and abolished inflammatory cell infiltration of the CNS [98]. A second EAE study demonstrated that inhibition of disease was due to a reduction of $CD4^+$ and macrophage infiltration to the spinal cord and an increase in IL-4, IL-10 and TGF-β cytokines indicating a shift from Th1 to Th2/Th3 cytokine response [99].

A multicenter, double-blind, randomized, placebo-controlled clinical trial in 209 RR-MS patients studied the safety, tolerability and efficacy on number of MRI lesions [100]. Patients were randomized to receive either placebo or 0.1 mg or 0.3 mg laquinimod administered orally in three tablets for 24 weeks. Patients receiving laquinimod 0.3 mg daily experienced a 44% reduction in new lesion formation measured by MRI compared with placebo groups. However, there was no observed difference in relapses rates or change in disability. The safety profile was favorable. A second double-blind, placebo-controlled, randomized, Phase IIb trial studied the effect of two doses of laquinimod (0.3 and 0.6 mg) in 306 patients over 36 weeks [101]. Patients receiving laquinimod 0.6 mg daily demonstrated a 40.4% reduction in the number of lesions compared with baseline levels. By contrast, patients receiving 0.3-mg treatment showed no effect. Treatment was well tolerated although some patients experienced transient increases in liver enzymes.

Fumaric acid esters

Fumaric acid esters (FAEs) are an unsaturated dicarboxylic acid in clinical use as second-line oral therapy for severe systemic psoriasis. FAEs are thought to have immunomodulatory properties, including the induction of Th2-type cytokines (IL-4, -5 and -10) [102,103], induction of apoptosis in activated T cells [102] and downregulation of cellular adhesion molecules (VCAM and ICAM) [104]. These properties suggest FAEs may be useful in the treatment of MS. A study in EAE demonstrated a protective effect of FAE when delivered by oral gavage twice daily [105]. A significant reduction in the number of infiltrating macrophages was observed in the spinal cord of the treated group and the blood levels of the protective cytokine IL-10 were increased [105].

An exploratory, open-label study of FAE was conducted in RR-MS patients where the end points were the number and volume of gadolinium-enhancing lesions, clinical outcomes measured by EDSS score and the ambulation index. Patients receiving FAEs demonstrated a significant decrease in lesion number and volume compared with baseline values and EDSS scores remained stable or slightly improved [106]. As with the EAE study, $CD4^+$ T cells from FAE-treated patients demonstrated higher levels of IL-10 secretion, and there was an increase in $CD4^+$ T-cell apoptosis. FAE was well tolerated and most adverse events were gastrointestinal symptoms, such as diarrhea, nausea and cramps. Four patients experienced a transient increase in liver enzyme levels, but all adverse events decreased over time.

Recently, a second-generation FAE derivative, BG00012, was developed to reduce the adverse effects observed with the first-generation FAEs [107]. A Phase II, double-blind, placebo-controlled trial was conducted to determine the efficacy of three doses (120, 350 or 720 mg daily) of FAE (BG00012) in 257 RR-MS patients compared with placebo over 24 weeks [108]. A reduction in the number of gadolinium-enhancing lesions was observed in patients receiving the high-dose FAE compared with placebo. A large-scale, Phase III trial is currently underway.

Monoclonal antibody therapy

The use of monoclonal antibodies – selective agents that can bind to and neutralize specific targets on the surface of cells or secreted cellular proinflammatory products – in the treatment of MS is one of the more promising therapeutic strategies being developed. The use of animal studies to further our understanding of the processes and cells involved in the pathology of MS has increased the number of potential disease-specific pathways we can target. Although the use of monoclonal antibodies can be advantageous owing to their unique specificity and capacity for industrial-scale production, they currently have some limitations due to the production of neutralizing antibodies,

injection-site reactions and serious side effects. An example of this is the use of anti-TNF antibody treatment in MS. Initial studies in EAE demonstrated the disease-promoting effect of TNF [109] and the protective effect of neutralizing TNF using either anti-TNF antibody [110–112], a p55 TNF-α-receptor–IgG_1 fusion protein (lenercept) [113] or gene-delivery of p75 dimeric TNF-receptor fusion protein [114]. Furthermore, studies in MS patients suggested a correlation between elevated levels of TNF in the serum and CSF of patients exhibiting disease activity [115–116]. Therefore, it was considered that TNF neutralization may be beneficial in the treatment of MS patients. Indeed, a number of clinical trials of anti-TNF-α therapy in patients with rheumatoid arthritis (RA), an inflammatory disease of the joints where TNF is a prime mediator, proved highly successful [117]. Evaluation of three doses of lenercept was undertaken in a double-blind, placebo-controlled, Phase II trial of 168 patients with RR-MS [118]. The primary clinical end point was a reduction of new MRI lesions. Patients received either 10, 50 or 100 mg of lenercept or placebo intravenously every 4 weeks up to 48 weeks in total. Although there was no difference between the treated or placebo groups in terms of numbers of new MRI lesions observed, patients receiving lenercept experienced disease exacerbations more frequently ($p = 0.007$) and earlier ($p = 0.006$) than the placebo group. Despite more severe neurological deficits in the lenercept-treated patients, there was no difference in the EDSS of either group at the end of the study. Since this study, a number of RA clinical trials have reported the presence of inflammatory demyelization events following treatment with various anti-TNF agents [119–121]. Why anti-TNF is deleterious in MS is not clear at present but may be due to the preparations of anti-TNF agent used. Alternatively, TNF is a pleiotropic cytokine and, therefore, its systemic neutralization may impact on other essential and protective functions.

Rituximab

Although MS is often thought to be a disease mediated by autoreactive T cells, there is increasing evidence that B cells also play a vital role in the immunopathogenesis of MS. The clonal expansion of B cells and the presence of oligoclonal IgG in the brain and CSF of people with MS is suggestive of their involvement in disease pathogenesis. The mechanism(s) by which B cells could mediate disease are unclear but could involve the antibody-mediated demyelination of CNS neurons or by providing cytokine help to encephalitogenic T cells. Studies by the group of Aloisi have demonstrated that lymphoid follicle-like structures containing B and T cells could be observed in the cerebral meninges of MS patients with SP-MS [122]. These findings suggest that B cells in the CNS could help maintain immune responses and exacerbate disease. Thus, B cells are increasingly seen as valid targets for the treatment of MS.

Rituximab is a human–mouse chimeric monoclonal antibody that targets the B-cell CD20 antigen and causes rapid and specific B-cell depletion. It has been licensed for the treatment of B-cell non-Hodgkin lymphoma resistant to other chemotherapy regimens by the FDA. Following three randomized, controlled trials, Rituximab, in combination with methotrexate, is also licensed for use in refractory rheumatoid disease [123]. A preliminary study of rituximab in four PP-MS patients demonstrated the depletion of peripheral, but not CSF, B cells although the activation state of these cells could be temporarily suppressed [124]. A study by Cross *et al.* demonstrated the ability of rituximab to deplete B and T cells in the CSF at 6 months post-treatment in RR-MS patients who were nonresponders to standard MS treatment, as measured by flow cytometry [125]. In addition, serum levels of antibodies to the myelin antigens, MBP and myelin oligodendrocytes protein measured by ELISA were shown to be reduced [125]. A Phase II, double-blind clinical trial in 104 RR-MS patients randomized to receive either 1000 mg rituximab intravenously ($n = 69$) or placebo ($n = 35$) on days 1 and 15 were followed for 48 weeks and the total count of gadolinium-enhancing lesions measured [126]. Other outcomes measured were safety, number of patients experiencing relapses and the annualized relapse rate. Patients receiving rituximab developed fewer total gadolinium-enhancing lesions (91% reduction; $p < 0.0001$) compared with placebo. In addition, patients receiving rituximab developed fewer relapses compared with placebo groups (14.5 vs 34.3% at week 24 and 20.3 vs 40.0% at week 48). Side effects were mild to moderate and usually appeared within 24 h of infusion.

Very recently, the FDA updated their public health advisory on the use of rituximab following the death of two patients treated with rituximab for systemic lupus erythematosus [202]. The patients died from progressive multifocal leukoencephalopathy associated with a life-threatening JC viral infection of the brain. Therefore, the use of potent immunosuppressive agents in the

treatment of MS and other diseases, such as systemic lupus erythmatosus, should be tempered with caution as the chance of fatal opportunistic infections appears to be increasing.

Alemtuzumab

Alemtuzumab (Campath-1H®) is a recombinant humanized monoclonal antibody that targets CD52, a protein present on the surface of mature lymphocytes, and is used in the treatment of chronic lymphocytic leukemia and T-cell lymphoma. Binding of alemtuzumab to CD52 induces complement or antibody-mediated lysis of T cells and their rapid and prolonged depletion from blood, bone marrow and peripheral organs.

Early pilot trials of alemtuzumab in small numbers of MS patients demonstrated rapid lymphopenia that was sustained for 1 year post-treatment, and also the reduction of the number and volume of gadolinium-enhancing lesions due to the suppression of active inflammation, although a decrease in brain volume was observed in some patients [127,128]. An open-label trial in 39 RR-MS patients with an aggressive form of disease observed a reduction in the mean annualized relapse rate of 2.48 to 0.19 following alemtuzumab treatment [129]. In addition, the mean change in EDSS was -0.36 overall and 83% of treated patients experienced stable or improved disability scores. Mild adverse events included rash and headache, although three patients developed a transient worsening of pre-existing deficits, which had also been observed in pilot studies [127]. In a study involving 58 RR-MS and SP-MS patients receiving alemtuzumab, there was a decrease in the annualized relapse rate (2.2–0.19 and 0.7–0.001, respectively) [130]. Although SP-MS patients demonstrated no new lesions following MRI measurement 7 years post-treatment, there was evidence of an increase in disability progression due to uncontrolled cerebral atrophy. By contrast, RR-MS patients experienced a reduction in disability [130]. This suggests that alemtuzumab treatment may facilitate the early rescue of neurons and axons from inflammation-induced damage in RR-MS compared with SP-MS where inflammation may be secondary to neurodegeneration. The CAMMS223 trial investigated the use of alemtuzumab in RR-MS patients randomized to receive either alemtuzumab or IFN-β_{1a} 44 µg subcutaneously three-times a week. After 2 years, the interim results demonstrated a significant reduction in the risk of relapse (75%) and reduction in the risk to progression of accumulated disability (65%) after alemtuzumab treatment compared with IFN-β treatment [131].

Daclizumab

Daclizumab (Zenapax®) is a therapeutic humanized monoclonal antibody to CD25, the α-subunit of the IL-2 receptor of T cells, and is licensed by the US FDA for prevention of graft-versus-host disease in renal transplant patients. Daclizumab has been tested in small MS trials with the rationale that blocking CD25, and therefore IL-2-mediated activation and stimulation of proliferation of T cells, would prevent the expansion of autoreactive T lymphocytes.

An open-label, baseline-to-treatment, Phase II trial in ten MS patients' refractory to standard MS treatments such as IFN-β demonstrated that it was well tolerated and patients experienced a 78% reduction in new gadolinium-enhancing lesions [132]. A second trial investigated the use of daclizumab in RR-MS patients who were refractory to IFN-β treatment and who still experienced relapses and new lesions measured by MRI [133]. Patients were kept on IFN-β treatment and received daclizumab at 1 mg/kg intravenously twice in the first month, then twice after another 2 weeks, then a treatment every 4 weeks. IFN-β treatment was continued until 5.5 months after initiation of daclizumab therapy, at which point daclizumab was continued as a monotherapy. However, patients experiencing exacerbation or new lesions were put back on IFN-β therapy and daclizumab was administered every 28 days. Only nine patients completed the trial and positive effects upon the number of new lesions, relapses, EDSS and neurologic scale were observed [133].

Antigen-specific/tolerance therapy

The induction of antigen-specific tolerance for MS treatment is attractive as it specifically targets the suppression of myelin-specific encephalitogenic T cells and should, therefore, spare T cells of other specificities so as not to leave the patient immunocompromised. This is of particular importance as multifocal leukoencephalopathy has been observed in clinical trials using potent immunosuppressive treatments.

Tolerance studies in EAE have proved highly successful in eliminating clinical disease following the administration of myelin autoantigens, altered peptide ligands (APLs) or DNA vaccination by a number of routes, including oral, nasal, intravenous and subcutaneous [134–139].

Based on the animal studies, a number of Phase II and III clinical trials were initiated in MS. However, these have by and large failed owing to severe adverse events or lack of efficacy. Oral tolerance has a number of advantages over other therapies, including the ease of

administration to patients and a favorable safety profile. However, a Phase III clinical trial treating patients with oral bovine MBP failed [140]. A trial to study the effect of intravenous administration of a soluble MHC complex loaded with MBP to induce tolerance in MBP-reactive T cells in the absence of costimulation, was shown to have a favorable safety profile, but had no clinical activity in SP-MS patients [141]. A MRI-controlled Phase II trial to test the tolerance effect of APL, an analog of the immunodominant MBP_{83-99} peptide that can induce a Th2 phenotype in T cells and induce bystander suppression, was also disappointing and was stopped as treatment induced relapses in three patients [142].

A 24-month, placebo-controlled, double-blinded Phase II clinical trial in 32 patients with progressive MS was undertaken to study the effect of a synthetic peptide MBP8298, with a sequence corresponding to amino acid residues 82–98 of human MBP which is immunodominant in MS patients with the HLA haplotype DR2, as a high-dose tolerance treatment for the long-term suppression of CSF levels of anti-MBP autoantibodies [143]. Doses of 500 mg of MBP8298 was administered intravenously every 6 months and changes in EDSS scores were measured. Patients with the DR2 or DR4 HLA haplotypes experienced significant increases in time to progression (78 months) compared with placebo group (18 months). Although anti-MBP autoantibody levels in the CSF of MBP8298-treated patients were suppressed, this was not indicative of clinical benefit [143].

A randomized, double-blind, placebo-controlled trial in 30 patients with RR-MS or SP-MS who were not on other disease-modifying drugs were assessed for the safety and efficacy of antigen-induced tolerance by BHT-3009, a DNA vaccine encoding full-length human MBP [144]. BHT-3009 was administered intramuscularly at 0.5, 1.5 or 3 mg on weeks 1, 3, 5 and 9, and patients were randomized to receive atorvastatin calcium 80 mg in combination. BHT-3009 treatment was well tolerated, reduced the number of MRI lesions and induced beneficial antigen-specific tolerance, measured by the reduction of proliferative responses of myelin specific IFN-γ-expressing $CD4^+$ T cells from the peripheral blood. In addition, titers of myelin-specific autoantibodies in the CSF were significantly reduced. A second Phase II clinical trial to test the efficacy and safety of BHT-3009 in 289 RR-MS patients over 44 weeks has recently been completed [145]. Patients were randomized to receive placebo, 0.5 or 1.5 mg BHT-3009 intramuscularly at weeks 0, 2 and 4, and every 4 weeks thereafter until week 44. Treatment with BHT-3009 0.5 mg inhibited new gadolinium-enhancing lesions by 50% during weeks 28 and 48, and 61% lower between weeks 8 and 48. Patients receiving BHT-3009 1.5 mg or placebo did not experience changes in lesion load.

To date, a universal target antigen for MS has not been described and, therefore, global tolerance therapy for MS remains unlikely in the near future. However, the association of certain HLA molecules with candidate myelin autoantigens suggests a proportion of patients may be amenable to tolerance therapy. Future studies must concentrate on the tailoring therapy to individual myelin responses. However, one confounding problem is that patients may respond to more than one myelin antigen and there is evidence to suggest that during the course of disease an individual's T-cell repertoire may become responsive to different epitopes, a phenomenon termed epitope spreading [146].

Hematopoietic stem cell transplantation

Hematopoietic stem cell transplantation (HSCT) is a new approach to treating autoimmune disease, with two distinct treatment arms. The first is an immunosuppressive phase where the aim is to suppress the 'disease-inducing' immune system, and the second is to reconstitute it with 'healthy immune cells' constituted from an infusion of autologous stem cells. Immunosupression can be achieved with chemotherapy agents such as carmustine, etoposide, cytosine-arabinoside, melphalan and cyclophosphamide, by total body irradiation or antithymocyte globulin, as reviewed by Muraro and Bielekova [147]. The source of stem cells for reconstitution can be obtained by mobilization of leukocytes from the bone marrow into the blood stream by G-CSF administration and then purified by $CD34^+$ selection or directly from bone marrow aspiration [147]. Another important aspect of HSCT is the source of the human stem cells. One source is autologous stem cells from the patient's own immune system; however, allogeneic stem cells could also be used and potentially may have higher efficacy as they would derive from a person with a healthy immune system rather than the patient's own.

A number of small-scale clinical studies (n = 14–26) in SP-MS, PP-MS and, to a smaller extent, RR-MS have been undertaken [148–154] and have demonstrated some efficacy as far as reducing the number of new MRI lesions (no new lesions in 72–100% patients) as well as reductions in the number of relapses. In addition, some

patients showed a trend for benefit on the EDSS with scores either stabilizing or showing some improvement after HSCT treatment. However, there were a number of deaths related to the treatment, which included complications due to infections such as aspergillosis [148], Epstein–Barr virus [149,154], pneumonia [153], varicella zoster virus hepatitis [153], or from neurological deterioration [150]. However, these results should be treated with caution as patients selected for trials were often diagnosed with SP-MS or PP-MS who do not experience relapses or new MRI lesions as often as RR-MS patients.

The results from a Phase I/II clinical trial of autologous nonmyeloablative HSCT in 21 RR-MS patients have recently been published. Patients were eligible for study if they had RR-MS and did not fully respond to IFN-β treatment exhibiting either two clinically definite relapses in 1 year or one relapse and new gadolinium-enhancing MRI lesions [155]. Autologous stem cells were mobilized with cyclophosphamide 2 g/m^2 and filgrastim 10 μg/kg daily from day 5. Mobilized stem cells were purified and cryopreserved. Following immune conditioning with intravenous cyclophosphamide 200 mg/kg and alemtuzumab 20 mg or rabbit antithymocyte globulin the hematopoietic stem cells were reinfused into patients. The primary end points were progression-free survival and a reversal of neurological disability. Patients were followed between 24 and 48 months and at a mean of 37 months 100% patients were progression free, 81% (17 of 21 patients) had improved by one point on the EDSS ($p < 0.0001$) and 76% were relapse free [155]. Further immunosuppression of the 24% of patients exhibiting relapse prevented further relapses occurring for the duration of the study. Side effects observed included dermatomal zoster infection and diarrhea due to *Clostridium difficile* infection. In addition, grade IV thrombocytopenia was observed but was due to the immune conditioning with alemtuzumab and, therefore, was changed to rabbit antithymocyte globulin treatment. The observed efficacy in this study hopefully means there may be great benefit from further testing HSCT in a double-blind, randomized, placebo-controlled clinical trial with larger numbers of patients.

As HSCT treatment may potentially reduce new lesion formation, the relapse rate and be effective at inhibiting neurological deficit in less progressive forms of MS disease, this treatment may be better suited towards patients with RR-MS where inhibition of the immune system can produce benefit and where neurodegeneration has not yet reached significant levels as is likely in SP-MS or PP-MS. However, as the risk level of HSCT still remains fairly high compared with other available treatments, this should be administered to those with a highly aggressive form of disease.

Future perspective

During the last decade, there have been numerous advances in the treatment of MS, culminating in six licensed treatments for RR-MS. However, these treatments are only partially effective. The use of MRI as a surrogate biomarker to monitor the pathology of disease and assess a patient's response to treatment has been vital for the diagnosis of MS and also to assess the value of first-line drugs in suppressing CNS inflammation during the early phase of disease. However, the use of MRI is of less value to predict future relapse episodes and identify neurodegeneration and during the later phase of disease. This has been demonstrated in many clinical trials where there is little correlation between the number and activity of MRI lesions and disability progression. Therefore, improvements in MRI and neuroimaging methodology may help measure the progression of disability and brain atrophy more accurately. This would greatly help facilitate clinical trials in RR-MS and the progressive forms of MS where there are currently no good clinical outcome measurements for neuroprotection.

Further refinement of our ability to diagnose disease earlier will augment the current thought that treatment should begin early during the diagnostic phase of disease to give it the best opportunity for success. The primary phase of RR-MS disease is thought to be inflammatory in nature and, therefore, early diagnosis and treatment at this time point may prevent secondary neurological damage due to chronic CNS inflammation.

Multiple sclerosis is increasingly thought of as having a late neurodegenerative phase and it has been suggested that the axonal injury caused by excessive inflammation in the CNS contributes to the irreversible neurological deficit seen in severe MS [146]. Therefore, although immunosuppressive agents are effective in the early inflammatory stage of disease, they are increasingly ineffective once neurodegeneration has begun. Novel strategies including neuroprotection using sodium channel blockers and the promotion of repair mechanisms utilizing neuronal stem cells, neuron growth factors and implantation of remyelinating cells are, therefore, currently being tested in clinical trials. Further elucidation of the pathogenesis of MS and continuing studies of the transition from relapsing

to more progressive forms of disease in terms of inflammation and neurodegeneration are vital to the future of MS therapy.

Multiple sclerosis is a heterogenous disease that can be divided into a number of subtypes and clinical trial data suggest that not all patients respond to a particular therapy in the same way. It seems increasingly likely that a 'one drug for all' and the 'trial and error' approach for therapy will not work in MS and that treatments will need to be personalized to achieve maximum efficacy. Further studies of responders versus nonresponders in clinical trials and the identification of disease parameters that determine therapeutic responses is an important issue that may help optimize treatments for certain forms of MS. In addition, the discovery of novel biomarkers of MS pathology, whether neurobiological, immunological or genetic, may allow a more targeted therapeutic approach to MS therapy.

The use of exciting new techniques, such as proteomics and genomics, in producing an individual's gene-expression profile with which to study the mechanisms of relapse or remission, subtypes of disease, responders versus nonresponders or poor responders, and biomarkers for disease and treatment, provides an optimistic future for the treatment of MS.

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Executive summary

- Multiple sclerosis is a complex heterogenous disease including relapsing–remitting and progressive subtypes of disease.
- While current therapeutic agents can suppress early inflammation, associated CNS lesion formation and relapses, the progression of disability is largely unaffected.
- Combination therapy with existing drugs is mostly less efficacious than monotherapy, has a less favorable risk-to-benefit ratio, and can lead to opportunistic infections, thus it should be used with caution.
- Novel therapeutic agents must treat both the inflammatory and neurodegenerative phases of disease whilst increasing the safety profile.
- Once disease progression has been suppressed, remyelinating and repair strategies using growth factors, stem cells and myelin-producing cells will be important.
- Current use of MRI is of benefit in diagnostics and measuring CNS lesion load, but is not predictive of future relapses or disability progression.
- Advanced neuroimaging technology may allow better analysis of candidate drugs' efficacy on neurodegeneration and neurological deficit.
- The use of new techniques, such as proteomics and genomics, may allow the discovery of new disease-related biomarkers for personalized treatment.

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Immunopathology and Infectious Diseases

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

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

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

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

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

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

Materials and Methods

Animals and Reagents

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

Induction and Evaluation of EAE

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

Preparation of Antibody and Fusion Protein

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

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

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

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

Histology

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

Flow Cytometry

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

In Vivo *Injection of α-GC*

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

Statistics

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

Results

CEACAM1 Has a Role in Ameliorating EAE

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

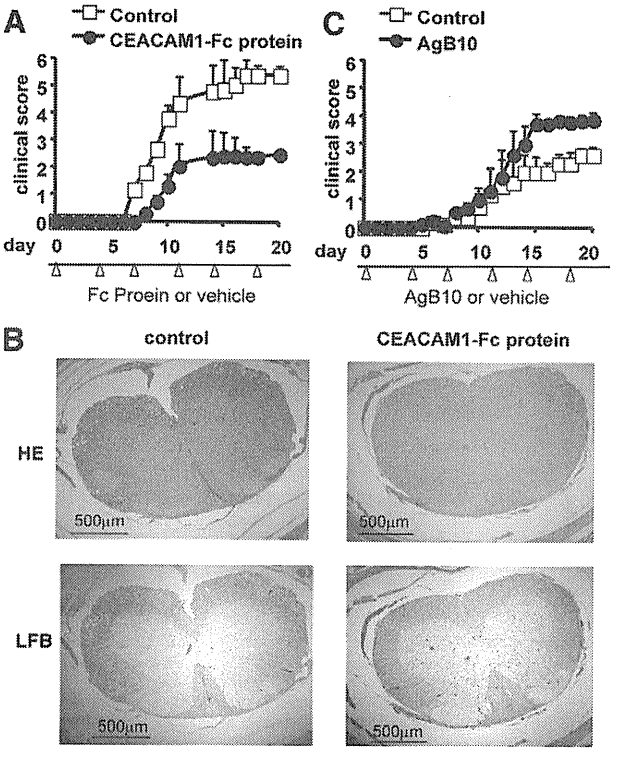

Figure 1. Effect of CEACAM1-Fc fusion protein or CEACAM1-specific antibody on MOG-induced EAE. EAE was induced in B6 mice by immunization with MOG_{35-55}. CEACAM1-Fc fusion protein (**A**) or a mAb for CEACAM1, AgB10 (**C**) was given twice per week starting from the day of immunization. **Arrowheads**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

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

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

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

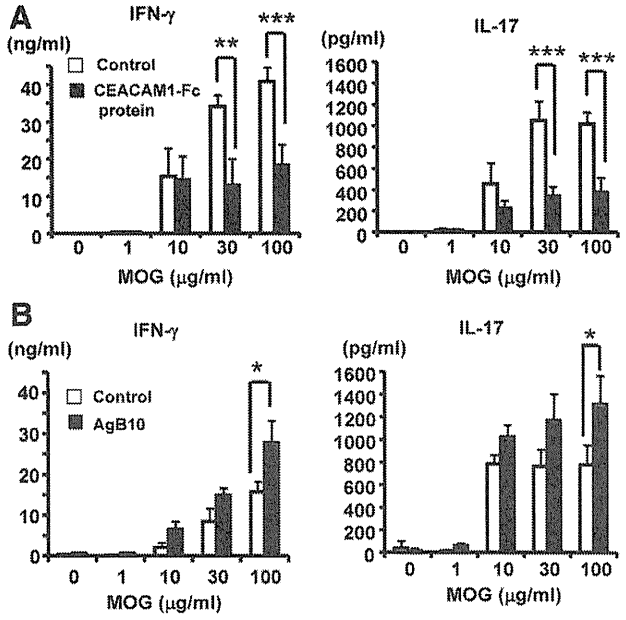

Figure 2. MOG_{35-55}-specific T cell responses in mice treated with CEACAM1-Fc fusion protein or with AgB10. B6 mice were treated with CEACAM1-Fc fusion protein (**A**) or AgB10 (**B**) twice per week from the day of immunization with MOG_{35-55}. Eleven days after the immunization, draining lymph node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

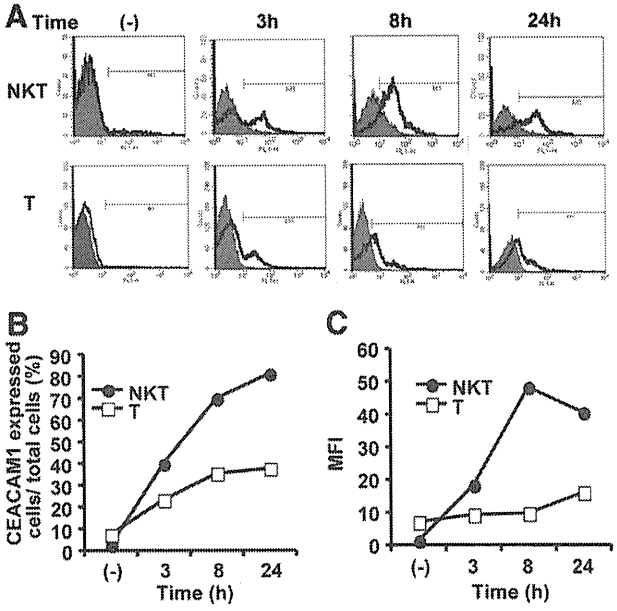

Figure 3. Expression of CEACAM1 on iNKT and T cells, after activation with ConA and anti-CD3 antibody. The histograms show the log fluorescence intensity of CEACAM1 on the surface of iNKT and T cells at the indicated time points after the activation with a combined treatment of ConA and plate bounded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

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

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

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

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

Rapid Expression of CEACAM1 on iNKT Cells after Activation

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

CEACAM1 Regulates IFN-γ Production from iNKT Cells

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

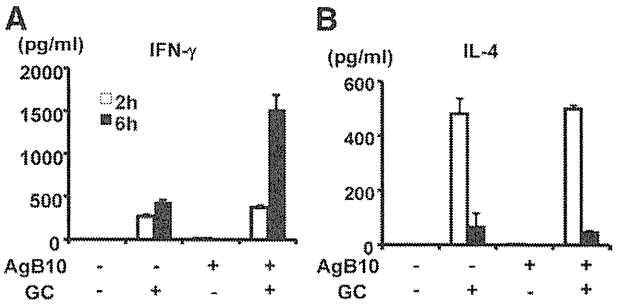

Figure 4. Effect of AgB10 on serum cytokine levels after *in vivo* injection with α-galactosylceramide (α-GC). B6 mice were treated with either AgB10 or control antibody. Four days after the treatment, serum was collected at 2 or 6 hours after intravenous injection of α-GC. Serum levels of IFN-γ and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

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 $J\alpha18^{-/-}$ 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 $J\alpha18^{-/-}$ mice, as compared with control mice (Figure 5A). To further determine the effect of the ligation of CEACAM1 on EAE in $J\alpha18^{-/-}$ mice, we analyzed the CNS inflammation and demyelination in EAE-induced $J\alpha18^{-/-}$ mice treated with CEACAM1-Fc fusion protein. In contrast to wild-type B6 mice, histological examination of the spinal cord of $J\alpha18^{-/-}$ mice showed cellular infiltration and demyelination to a similar extent as sham-treated mice (Figure 5B). We next induced EAE in $J\alpha18^{-/-}$ mice treated with either AgB10 or control antibody. Again, no suppression of clinical EAE was observed in AgB10- treated $J\alpha18^{-/-}$ mice, as compared with the control mice (Figure 5C).

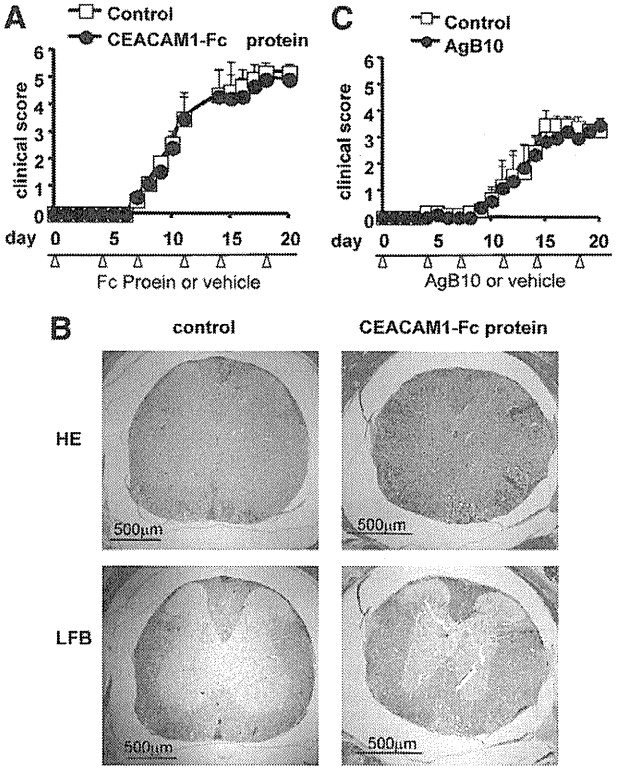

Figure 5. Effect of CEACAM1-Fc fusion protein or CEACAM1-specific antibody on EAE induced in $J\alpha18^{-/-}$ mice. EAE was induced in $J\alpha18^{-/-}$ mice by immunization with MOG_{35-55}. CEACAM1-Fc fusion protein (**A**) or AgB10 (**C**) was given twice per week starting from the day of immunization. **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 $J\alpha18^{-/-}$ 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 $J\alpha18^{-/-}$ 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 $J\alpha18^{-/-}$ 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 $J\alpha18^{-/-}$ mice with AgB10 also did not significantly enhance of MOG_{35-55}-specific T cell IFN-γ and IL-17 production (Figure 6B).

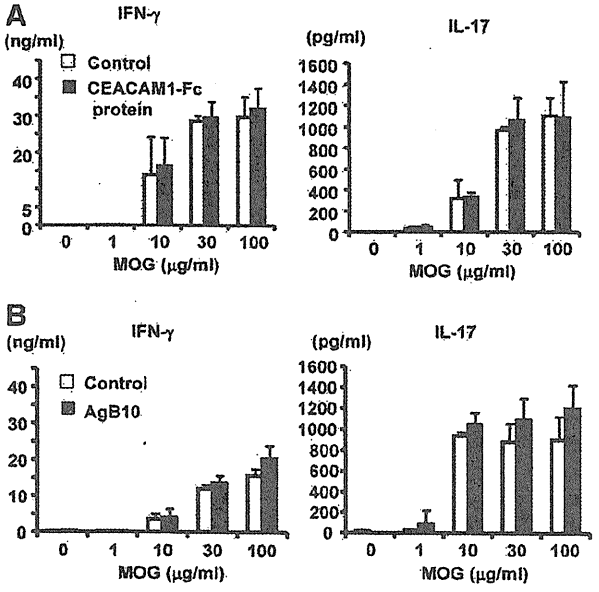

Figure 6. MOG_{35-55}-specific T cell responses in $J\alpha18^{-/-}$ mice treated with CEACAM1-Fc fusion protein or with AgB10. $J\alpha18^{-/-}$ mice were treated with CEACAM1-Fc fusion protein (A) or AgB10 (B) twice per week from the day of immunization with MOG_{35-55}. Eleven days after the immunization,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

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

Discussion

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

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

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

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

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