Cooperation of Invariant NKT Cells and CD4+CD25+ T Regulatory Cells in the Prevention of Autoimmune Myasthenia

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CD1d-restricted NKT cells and CD4\textsuperscript{+}CD25\textsuperscript{+} regulatory T (Treg) cells are thymus-derived subsets of regulatory T cells that have an important role in the maintenance of self-tolerance. Whether NKT cells and Treg cells cooperate functionally in the regulation of autoimmunity is not known. We have explored this possibility in experimental autoimmune myasthenia gravis (EAMG), an animal model of human myasthenia gravis, induced by immunization of C57BL/6 mice with the autoantigen acetylcholine receptor. We have demonstrated that activation of NKT cells by a synthetic glycolipid agonist of NKT cells, \(\alpha\)-galactosylceramide (\(\alpha\)-GalCer), inhibits the development of EAMG. \(\alpha\)-GalCer administration in EAMG mice increased the size of the Treg cell compartment, and augmented the expression of foxp3 and the potency of CD4\textsuperscript{+} T cells. Furthermore, \(\alpha\)-GalCer promoted NKT cells to transcribe the IL-2 gene and produce IL-2 protein. Depletion of CD25\textsuperscript{+} cells or neutralization of IL-2 reduced the therapeutic effect of \(\alpha\)-GalCer in this model. Thus, \(\alpha\)-GalCer-activated NKT cells can induce expansion of CD4\textsuperscript{+}CD25\textsuperscript{+} Treg cells, which in turn mediate the therapeutic effects of \(\alpha\)-GalCer in EAMG. Induced cooperation of NKT cells and Treg cells may serve as a superior strategy to treat autoimmune disease. The Journal of Immunology, 2005, 175: 7898–7904.

Characterization of CD1d-restricted invariant NKT cells in humans with autoimmune disease and autoimmune-prone mouse strains has suggested that defective NKT cell function relates to the emergence of autoimmunity (1, 2). In many experimental models of autoimmunity (e.g., type 1 diabetes, encephalomyelitis, etc.), intentional activation of NKT cells by a synthetic glycolipid agonist, \(\alpha\)-galactosylceramide (\(\alpha\)-GalCer), can elicit the regulatory functions of NKT cells and prevent autoimmunity (3). Induction of Th2 deviation and the generation of tolerogenic dendritic cells (DCs) have been suggested as mechanisms governing the protective function of NKT cells in these models (2). However, induction of Th2 deviation or generation of tolerogenic DC has not been demonstrated in all model systems tested to date. In addition, Th2 deviation in response to therapeutic intervention is sometimes an outcome rather than the cause of disease protection (2). Therefore, additional mechanisms underlying the regulatory role of NKT cells in autoimmune disease must be involved.

The unique features of NKT cells are reminiscent of another T regulatory (Treg) cell population: CD4\textsuperscript{+}CD25\textsuperscript{+} Treg cells. Both NKT cells and CD4\textsuperscript{+}CD25\textsuperscript{+} Treg cells are thymus-derived subsets of regulatory T cells that have an important role in the maintenance of self-tolerance. Whether NKT cells and Treg cells cooperate functionally in the regulation of autoimmunity is not known. We have explored this possibility in experimental autoimmune myasthenia gravis (EAMG) induced by immunization of C57BL/6 (B6) mice with the autoantigen acetylcholine receptor (AChR). EAMG serves as an animal model of human myasthenia gravis (MG), which is a classic T cell-driven, autoantibody (against AChR)-mediated disorder of neuromuscular junctions (4, 5). In this study, we demonstrate that \(\alpha\)-GalCer-activated NKT cells can induce expansion of CD4\textsuperscript{+}CD25\textsuperscript{+} Treg cells, which in turn mediate the therapeutic effects of \(\alpha\)-GalCer in EAMG.

Materials and Methods

**Mice**

C57BL/6 (B6) mice and IL-4-deficient mice (6) on the B6 background were purchased from The Jackson Laboratory. CD1d-deficient mice (7) (lacking invariant NKT cells) were generated as described and were backcrossed to the B6 background for 11 generations. Female mice, aged 8–10 wk at the initiation of the experiments, were used. Animal experimental procedures were in compliance with institutional guidelines.

**Age and peptide**

*Torpedo* AChR (TAChr) was purified from the electric organs of *Torpedo californica* (Pacific Bio-Marine) by affinity chromatography on a \(\alpha\)-cobratoxin-agarose resin (Sigma-Aldrich) (8). Mouse AChR was purified from mouse muscle tissues, as described (5). The isolated product was pure as judged by SDS-PAGE. The AChR \(\alpha1\)–162 (LGIWTYDGTKVSISPES) peptide (8) was synthesized at Biosynth International.
Induction and clinical evaluation of EAMG

Mice were immunized s.c. with 20 µg of TACRh in CFA in a total volume of 100 µl, along the shoulders and back. Mice were boosted once after 1 mo with 20 µg of ACHR in IFA s.c. at four sites on the shoulders and thighs (8). The mice were observed every other day in a blinded fashion for signs of muscle weakness characteristic of EAMG. Clinical manifestation of EAMG was graded between 0 and 3 (8): 0, no definite muscle weakness; 1+, normal strength at rest, but weak with chin on the floor and inability to raise the head after exercise consisting of 20 consecutive pawgrips; 2+, as grade 1+ and weakness at rest; and 3+, moribund, dehydrated, and paralyzed. Clinical EAMG was confirmed by injection of neostigmine bromide and atropine sulfate.

Treatment of mice with α-GalCer

A synthetic form of α-GalCer, KNR7000, was obtained from Kirin Brew- ery for this study. Three injections of α-GalCer (6 µg/mouse/injection) or vehicle (0.025% polysorbate-20 in PBS) were performed on days 0, 4, and 7 after immunization with TACRh. To determine the efficacy of α-GalCer on clinical EAMG when autoimmunity against ACHR is induced and muscular weakness becomes present, the same amount of α-GalCer or vehicle was injected on days 30, 34, and 37 after immunization with ACHR.

FACS analysis

For flow cytometry analysis, single cell suspension (4 × 10^6 or 10^6 cells) was prepared and stained with fluorescently labeled Abs. All Abs were purchased from BD Pharmingen, unless otherwise indicated. Abs were directly labeled with one of the following fluorescent tags: FITC-, PE-, PerCP-, allophycocyanin-, PC-, or PC7-: -NK1.1 (PK136), -CD3 (145–2C11), -CD4 ( GK1.2), -CD11c (HI.104), -I-A/K (H-2D), -CD69 (H.2F3), -CD80 (16–10A), -CD86 (GL-1), -Bcl-2 (6C8), -TCR-β (H-57–597). Foxp3 staining was performed with PE anti-mouse foxp3 (clone FJK-16s) (eBioscience), following the manufacturer’s instructions. Isotype control for foxp3 IgG2a was used. All samples were analyzed on a FACSaria or FACSCalibur using Diva or CellQuest software.

In vivo Ab treatment

For depletion of CD25+ T cells, 250 µg of anti-CD25 Ab (clone PC61; BD Pharmingen) (9) was injected i.v. into mice on day 0. A total of 100 µg of such Ab was injected every 5 days to maintain the depletion efficacy until termination of experiments. Control mice received the same amount of rat IgG control Ab (Sigma-Aldrich). The rat anti-IL-2 mAb (IgG2a, clone S4B6) (10) was purified from ascites fluid using protein G columns (Am-ersham Biosciences). A total of 150 µg of anti-IL-2 mAb or control Ab rat IgG2a (Sigma-Aldrich) was injected i.v. for initial neutralization. Same amount of anti-IL-2 mAb was injected i.v. every 5 days for long-term neutralization.

Cell purification

We incubated single spleen cell suspensions with biotin anti-CD25 Ab, followed by streptavidin microbeads. CD25 T cells were then purified by using an AutoMACS sorter. In all experiments, 90–95% of these cells were positive for both the CD4 and CD25 markers. NKT cells (NK1.1+CD4+CD8+) were sorted by FACSaria with Diva software, with the purity >98%.

Treg cell suppression assays

CD4+CD25+ cells were used as responders. We cultured CD4+CD25+ T cells (1 × 10^5) in U-bottom 96-well plates with 10^4 spleen DCs, TACRh, ACHR α146–162 (10 µg/ml), anti-CD3 (0.1 µg/ml), and the indicated numbers of CD4+CD25+ T cells from control or α-GalCer-treated EAMG mice for 60 h. CD11c+ cells were sorted from splenocytes of EAMG mice at day 3–7 postinfection (p.i.) and further loaded with ACHRα146–162 peptide ex vivo. These DCs were more potent than DCs from naive mice in generating measurable T cell response (data not shown). Proliferation was determined by incorporation of [3H]thymidine for the 18 h of the culture.

Lymphocyte proliferation responses

Triplicate aliquots (200 µl) of mononuclear cell suspensions containing 4 × 10^6 cells were placed into 96-well round-bottom microtiter plates (Nunc). Ten-microliter aliquots of TACRh, α146–162, or Con A (5 µg/ml) (Sigma-Aldrich) were added in triplicate into appropriate wells. After 4 days of incubation, the cells were pulsed with 18 h with 10-µl aliquots containing 1 µCi of [3H]thymidine (sp. act. of 42 Ci/mmol; Amer- sham). Cells were harvested onto glass fiber filters, and thymidine incor- poration was measured. The results are expressed as cpm.

Cytokine ELISA

Single cell suspensions of TACHr-primed draining lymph node cells were cultured in the presence or absence of TACHr (10 µg/ml). The superna- tants were collected after 48 h in culture. IFN-γ, IL-12, and IL-4 produc- tion in culture supernatants was measured by optEIA kits (BD Pharmin- gen). For IL-2, we used a capture Ab JES6-1A12 and detection Ab JES6-54H.

Measurement of anti-AChR IgG Abs

Anti-TACHr IgG Abs and anti-murine ACHR IgG Abs were detected by ELISA, as described previously (5, 8). Microtiter plates (Corning Glass) were coated with 100 µlwell TACHr (2 µg/ml) or murine ACHR (0.5 µg/ml) at 4°C overnight. Uncoated sites were blocked with 10% FCS (In- vitrogen Life Technologies). Serum samples were added and incubated for 2 h at room temperature. Then plates were incubated with biotini- lated rabbit anti-mouse IgG, IgG1, and IgG2b (1/200; Dakopatts), 1/100 for IgG2a, followed by alkaline phosphatase-conjugated ABC (Dakopatts). The color was developed with p-nitrophenyl phosphate. Results were expressed as OD at 405 nm.

RT-PCR

Total RNA was extracted from purified NKT cells and was reverse transcribed into cDNA using SuperScript II reverse transcriptase and oli- go(dT)12–18 primer (Invitrogen Life Technologies). The relative quantity of CDNA in each sample was first normalized after semi quantitative PCR for hypoxanthine phosphoribosyltransferase (HPRT), Reaction mixture (40 µl) contained 1.5 mM MgCl2, 0.2 mM dNTP, 0.5 µM forward and reverse primer, and 1.0 U of TaqDNA polymerase (Promega) in the supplier’s buffer. PCRs were performed on a PTC-200 Programmable Thermal Con- trolled System (BioRad). For HPRT amplifications, PCRs consisted of a 2.5- min at 94°C denaturation step, followed by 28 cycles of 30 at 94°C, 30 s at 60°C, and 30 s at 72°C. IL-2 mRNA levels were also quantified by real-time PCR using the ABI/PRISM 700 sequence detection system (Ap- plied Biosystems). Analyses were performed using primers, an internal fluorescent TaqMan probe specific to IL-2 or HPRT, and the QuantTect Probe PCR Kit (Qiagen). PCRs contained 0.4 µM primers and 0.2 µM TaqMan probe, and consisted of a 10 min at 95°C denaturation step, fol- lowed by 40 cycles of 15 s at 95°C and 60 s at 60°C. The primer and TaqMan probe sequences were as follows. Normalized value for IL-2 mRNA expression in each sample was calculated as the relative quantity of IL-2 divided by the relative quantity of HPRT (×100). All samples were run in triplicate (10). The IL-2 primer sequences were 5′-tcagcaagattgcccag-3′ and 5′-ctgtgagcagagagataatac-3′. The IL-2 probe used was 5′- FAM-actccccaggatgctaccttcaaattt-3′.

In vivo BrdU labeling

Mice were injected i.p. with 1.0 mg of BrdU (Sigma-Aldrich) every 12 h for 3 days. Single cell suspensions were surface stained with CD4, CD25, and FITC-labeled anti-BrdU mAb (DakoCytomation).

Lymphocyte labeling with CFSE

Purified lymphocytes were incubated with CFSE at a concentration of 0.5 µM at 37°C for 30 min. Cells were washed and seeded in plates at a concentration of 4 × 10^5 cells/well. Cells were incubated with the following stimuli: ACHR (10 µg/ml), α-GalCer (10 µg/ml), or Con A (5 µg/ml). After 72 h of incubation, the cells were washed, stained with surface Abs to CD4 and CD25, and analyzed by FACS.

Statistical analysis

Differences between groups were analyzed by Student’s t test. Clinical scores were analyzed using the nonparametric Mann-Whitney U test. Differences between the groups with respect to disease incidence were ana- lyzed by Fisher’s exact test. The level of significance was set at p < 0.05.

Results

α-GalCer therapy alleviates the symptoms of EAMG

We have previously reported that NKT cell-deficient Jα281−/− (11) and CD1d−/−/− (7) mice, upon immunization with TACHr/ CFA, develop EAMG at a magnitude similar to that of their cor- responding wild-type controls (12). These results indicated that NKT cells by themselves do not significantly affect the development of EAMG. To assess the role of α-GalCer-activated NKT cells in the development of TACHr-induced EAMG, B6 mice...
were immunized with TACHR in CFA and treated with α-GalCer (KRN7000) or vehicle obtained from Kirin Brewery. For this study, α-GalCer (6 μg/mouse/injection) or vehicle (0.025% polysorbate-20 in PBS) was injected on days 0, 4, and 7 after immunization with TACHR (13). α-GalCer treatment at the time of primary immunization delayed the onset of EAMG (day 18 p.i. vs day 35 p.i. in control mice) and suppressed the severity of muscle weakness (p < 0.01 after day 32 p.i.; Fig. 1a). Next, we evaluated whether α-GalCer therapy can lessen muscular weakness and halt the progression of EAMG. For this purpose, α-GalCer treatment was initiated on days 30 after immunization with TACHR, a time point at which EAMG becomes progressive. As shown in Fig. 1b, α-GalCer therapy again significantly ameliorated the symptoms of EAMG (p < 0.04 after day 50 p.i.). However, the efficacy of this delayed α-GalCer delivery was reduced (severity of muscular weakness: p = 0.038 at day 85 p.i.). Thus, α-GalCer therapy alleviates the symptoms of EAMG, and this effect is more dramatic when treatment is initiated during the induction phase of EAMG.

Effects of α-GalCer therapy on cellular and humoral immune responses to AChR

In MG and EAMG, the production of anti-AChR Abs is T cell dependent (4, 5). Thus, the reduction of anti-AChR Abs could be secondary to altered Th cell functions after α-GalCer treatment. T cell proliferative responses to AChR may reflect expansion of autologous T cells and may relate to disease severity (4). Therefore, we examined T cell responses in TACHR-immunized mice treated with α-GalCer or vehicle. Compared with vehicle-treated mice, α-GalCer-treated mice showed reduced proliferation of T cells in response to TACHR and a major determinant, α146–162, on the α subunit (Fig. 1c). However, treatment with α-GalCer did not alter T cell proliferation upon Con A stimulation, indicating that strong T cell stimuli can bypass the suppressive effects of α-GalCer. Production of IFN-γ in response to TACHR was also suppressed by α-GalCer treatment. In contrast, α-GalCer treatment did not significantly alter TACHR-induced IL-4 production (Fig. 1d), indicating lack of Th1/Th2 shift.

The pathogenic anti-AChR Abs in MG and EAMG are predominantly IgGs (4, 8). To address how anti-AChR Ab responses are influenced by α-GalCer treatment, we measured the levels of TACHR and murine AChR-reactive IgG in serum. Mice that received α-GalCer during EAMG induction had levels of anti-TACHR and murine AChR IgG and IgG2b about one-half of that of control mice, and levels of TACHR and murine AChR-reactive IgG1 and IgG2a were comparable in the two groups. Compared with control mice, mice given α-GalCer during EAMG progression also had reduced levels of anti-TACHR and murine AChR IgG and IgG2b (Fig. 1, e and f). The capacity of α-GalCer to suppress the anti-AChR Ab response may underlie its clinically beneficial effects on EAMG.

The therapeutic effects of α-GalCer in EAMG do not entirely depend on IL-4

To attribute the therapeutic effects of α-GalCer to the activation of NKT cells, we treated NKT cell-deficient CD1d<−/−> mice with α-GalCer. None of the disease parameters was significantly different among the untreated CD1d<−/−> and control mice, indicating that α-GalCer failed to protect CD1d<−/−> mice from EAMG (Table I). These results provide strong evidence that α-GalCer prevents the development of EAMG in mice by the CD1d-dependent activation of NKT cells.

FIGURE 1. Clinical EAMG and immune response to AChR in mice treated with α-GalCer. EAMG was induced in B6 mice, and these animals were then treated with α-GalCer or vehicle. α-GalCer administration during EAMG induction (a) or during EAMG progression (b) alleviated symptoms of disease. Mice depicted in a were sacrificed on day 85 p.i. Splenocytes from TACHR/CFA-primed mice were isolated and cultured for 3 days. c, Proliferative responses of splenic T cells to TACHR and AChR α146–162 peptide were assessed by a [14H]thymidine uptake assay (n = 8). d, Cytokine production in response to the indicated stimuli was quantified by ELISA (n = 8). The spontaneous cytokine release was 37 ± 23 pg/ml for IFN-γ and IL-4 undetectable. e, Anti-TACHR IgG Ab and IgG isotypes were analyzed by ELISA (n = 10). f, Anti-murine AChR IgG Ab and IgG isotypes were analyzed by ELISA (n = 10). All results are expressed as mean values ± SD. *, p < 0.05.
Production of IL-4 by activated NKT cells has been proposed to be responsible for the protective effects of NKT cell activation against autoimmune diseases (2). To evaluate the role of IL-4 in mediating the effects of α-GalCer in EAMG, we tested the ability of α-GalCer to modulate disease in B6.IL-4−/− mice. Although published reports regarding the phenotype of EAMG in IL-4−/− mice are conflicting (14, 15), in our hands, disease severity and incidence were comparable in IL-4−/− and control mice (Table I). Nonetheless, α-GalCer therapy was still effective in alleviating EAMG in IL-4−/− mice, albeit less pronounced (but not significantly different) than in wild-type mice (Table I). Thus, IL-4 deficiency only partially reduced the therapeutic effect of α-GalCer in EAMG.

Activated NKT cells from EAMG mice transcribe the IL-2 gene and produce IL-2

NKT cells have been studied extensively for their ability to produce either Th1 or Th2 cytokines (IL-4, IL-10, IL-13, TNF-α, and IFN-γ) after activation, which are suggested to be responsible for their immunoregulatory function in vivo (2). We chose to determine IL-2 expression by α-GalCer-activated NKT cells because of the potential role of this particular cytokine in the induction and maintenance of Treg cells (10, 16). As Fig. 2 demonstrates, IL-2 mRNA expression was 3- to 4-fold higher in NKT cells from α-GalCer-treated EAMG- than those from vehicle-treated EAMG mice (Fig. 2, a and b). Furthermore, NKT cells from α-GalCer-treated EAMG mice produced large amounts of IL-2, 4- to 5-fold higher than NKT cells from vehicle-treated EAMG mice (Fig. 2c). Collectively, our results indicate that α-GalCer-activated NKT cells from EAMG mice transcribe the IL-2 gene and abundantly produce IL-2 upon activation.

α-GalCer administration induces expansion of CD4+CD25+ Treg cells in EAMG

Because the therapeutic effect of α-GalCer does not entirely depend on the Th2 cytokine IL-4, an alternative mechanism must be operating. One possibility is that the α-GalCer-activated NKT cells act via induction of CD4+CD25+ Treg cells. To test this possibility, we first quantified the number and frequency of CD4+CD25+ Treg cells from EAMG mice given α-GalCer or vehicle. Compared with the vehicle-injected EAMG mice, α-GalCer-treated recipients had ∼1.5- to 2-fold increased numbers and percentages of CD4+CD25+ cells in the spleens, which was observed as early as 5 days after immunization and α-GalCer treatment (Fig. 3a). The expansion of CD4+CD25+ Treg cells in α-GalCer-treated mice appeared to result from cell in situ proliferation rather than recruitment, because the numbers of CD4+CD25+ cells were similarly increased in the other organs examined (Fig. 3b). α-GalCer also induced expansion of Treg cells in spleen, lymph node, and blood of thymectomized EAMG mice (Fig. 3a and data not shown), indicating that the effects of α-GalCer are independent of the thymus. Treg cell numbers were comparable in vehicle- and α-GalCer-treated CD1d−/− mice (Fig. 3a), indicating that NKT cells are responsible for Treg cell expansion.

We asked whether Treg cells induced α-GalCer-activated NKT cells to acquire unique features, and we determined expression of an antiapoptotic factor bcl-2 and foxp3, a transcription factor that controls the development and function of Treg cells (16, 17). Treg cells from α-GalCer-treated EAMG mice expressed higher levels of anti-apoptotic Foxp3 and bcl-2 (Fig. 4, a and b). To compare the proliferation of Treg cells between control mice and α-GalCer-treated EAMG mice, we determined the incorporation of BrdU by

Table I. The therapeutic effect of α-GalCer occurs via CD1d1-restricted NKT cells and is not significantly altered by IL-4 deficiency

<table>
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<tr>
<th>Groups of Mice</th>
<th>No. of Mice</th>
<th>Treatment</th>
<th>Onset of Disease (day)</th>
<th>Clinical Score ± SD</th>
<th>Disease Incidence (%)</th>
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<tr>
<td>1. C57BL/6</td>
<td>8</td>
<td>Vehicle</td>
<td>24.4 ± 5.8</td>
<td>2.7 ± 0.9*</td>
<td>68</td>
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<td>2. C57BL/6</td>
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<td>α-GalCer</td>
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<td>3. CD1d−/−</td>
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<td>27.7 ± 6.5</td>
<td>2.7 ± 0.6</td>
<td>65</td>
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<td>4. CD1d−/−</td>
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<td>α-GalCer</td>
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<td>2.6 ± 0.7</td>
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<td>5. IL-4−/−</td>
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<tr>
<td>6. IL-4−/−</td>
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<tr>
<td>7. IL-4−/−</td>
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<td>Vehicle</td>
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<td>2.9 ± 0.9</td>
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<tr>
<td>8. IL-4−/−</td>
<td>16</td>
<td>α-GalCer</td>
<td>31.0 ± 5.0</td>
<td>2.0 ± 0.7</td>
<td>53</td>
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</table>

*EAMG was induced in B6 mice, B6 CD1d−/− mice, and B6 IL-4−/− mice by immunization with purified TACHR and CFA. Mice were treated with α-GalCer (6 μg/injection) or vehicle (0.025 polysorbate-20 in PBS) on days 0, 4, and 7, and disease was monitored, as described in Materials and Methods. *, p < 0.05.
these cells. Treg cells from α-GalCer-treated EAMG mice proliferated more abundantly than those from vehicle-injected EAMG mice (Fig. 4b).

Next, we determined proliferation of Treg cells by incorporation of BrdU. Treg cells from α-GalCer-treated EAMG mice proliferated more than those from vehicle-injected EAMG mice (Fig. 4b). To further study the ability of Treg cells to proliferate upon stimulation with α-GalCer, we studied Ag-specific (to AChR) or polyclonal (Con A) responses of splenocytes from treated mice vs controls. CFSE-labeled gated CD4^+CD25^+ cells from α-GalCer-treated EAMG mice displayed basal proliferative responses that increased further upon Ag-specific or polyclonal stimulation (Fig. 4c).

CD4^+CD25^+ Treg cells from α-GalCer-treated mice suppress CD4^+CD25^− T cells

In an attempt to link the up-regulation of bcl-2 and foxp3 and hyperproliferative property of Treg cells in α-GalCer-treated mice with Treg cell functions, we assessed the suppressive potential of CD4^+CD25^+ Treg cells in EAMG. CD4^+CD25^+ cells from naive or EAMG mice did not respond to anti-CD3 Ab stimulation, whereas CD4^+CD25^+ cells from these mice proliferated vigorously in response to anti-CD3 Ab stimulation, as reported (18). In coculture experiments, CD4^+CD25^+ cells inhibited proliferation of CD4^+CD25^− cells ~3-fold, confirming their suppressive activity. CD4^+CD25^+ cells from α-GalCer-treated EAMG mice were also more potent in inhibiting responses of CD4^+CD25^− cells to TACHR and AChRox146–162 stimulation when compared with vehicle-injected EAMG mice (Fig. 5). In some of these experiments, we used highly purified CD4^+CD25^+ cells (>98% sorted by FACSaria) and produced comparable results (data not shown). These data suggest that CD4^+CD25^+ cells in α-GalCer-treated mice modulate autoimmune reactivity in these mice.

CD4^+CD25^+ Treg cells contribute to the therapeutic effects induced by α-GalCer

The data presented to date suggest that activated NKT cells induce Treg cells, which are potent in inhibiting autoreactive T cells. To investigate whether these Treg cells indeed contribute to the therapeutic effects induced by α-GalCer, we compared the effects of anti-CD25 or control Ab on the development of EAMG in α-GalCer-treated mice. In preparatory experiments, we have demonstrated that anti-CD25 mAb by itself did not dramatically affect the Ag-activated T cells and clinical EAMG (Table II). This might be due to either the limited number of activated T cells expressing...
CD25 in this system (see above) or additional factors that can intervene in the ultimate outcome.

Compared with mice receiving control Ab, anti-CD25 Ab treatment largely diminished the therapeutic effects of α-GalCer on clinical EAMG and T and B cell responses to AChR (Table III). Importantly, neutralization of IL-2 also reduced the therapeutic efficacy of α-GalCer in EAMG (Table III). These data strongly suggest that the therapeutic effect of α-GalCer is via collaboration of NKT cells and Treg cells, and that IL-2 bridges the interactions between NKT cells and Treg cells.

Discussion
Many studies have addressed the capacity of Treg cells to suppress effector T cells in various pathological conditions, including inflammation, autoimmunity, cancer, and organ transplantation (16, 18–20). In contrast, few studies have examined whether Treg cells are subjected to regulation by other cells. In this study, we have demonstrated that Treg cell functions during the autoimmune disease EAMG are controlled by another regulatory T cell subset, NKT cells. α-GalCer-activated NKT cells promote the development/expansion and function of Treg cells, in a mechanism in-between NKT cells and Treg cells.

α-GalCer-activated NK cells after activation of autoreactive T cells may not serve as a primary source of IL-2. The mechanisms of action of this cytokine, namely induction of Treg cells and prevention of autoimmunity. Treg cells from α-GalCer-treated EAMG mice, at the same number of Treg cells from vehicle-treated mice, are more potent to suppress autoreactive T cells. Factors in addition to IL-2 and Treg cell expansion may also contribute to the enhanced functions of Treg cells. The combined effects of up-regulating bcl-2, foxp3, and IL-2 induced by α-GalCer-activated NKT cells would underlie the enhanced functions of Treg cells.

The emergence and progression of autoimmunity to a degree that the normal function of target organs is being disturbed must overcome the suppressive function of regulatory cells, including CD4⁺CD25⁺ Treg cells and NKT cells. Indeed, an elegant study by Pasare and Medzhitov (9) has indicated that microbial induction of the Toll pathway blocks the suppressive effect of CD4⁺CD25⁺ Treg cells, allowing activation of pathogen-specific adaptive immune responses. Humans with autoimmune diseases such as MG and multiple sclerosis (MS) often have defective Treg cell functions (22–24). Therefore, strategies to restore and enhance one or several types of regulatory cells would have significant implications for improving the efficacy of current immunomodulatory drugs in autoimmune disorders. Glatiramer acetate (Copaxone, Copolymer-1) is a Food and Drug Administration-approved drug for treatment of relapse-remitting MS. The mechanism of action of this copolymer is a matter of debate. Emerging evidence suggests that the copolymer may restore defective CD4⁺CD25⁺ Treg cell functions in MS patients by augmenting foxp3 expression (23–26). α-GalCer therapy may represent a superior strategy because it can stimulate both NKT cells and Treg cells and induce functional cooperation between these cells to disrupt the pathogenic responses in autoimmune disease. It may be possible to enhance the therapeutic properties of NKT cells and Treg cells by combined treatment with α-GalCer and copolymer. This possibility is currently being explored in our group.

We have observed that the efficacy of α-GalCer in alleviating the symptoms of EAMG becomes less dramatic when treatment is initiated after development of muscular weakness. There are several possible explanations for this observation. First, reversal of disease requires both depletion of existing anti-AChR Abs and prevention of the generation of new anti-AChR Abs. α-GalCer therapy would be only effective in suppressing new Ab production. Second, IL-2 produced by α-GalCer-activated NK cells after activation of autoreactive T cells may not serve as a primary source

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**Table II. Anti-CD25 mAb treatment does not significantly alter T cell responses to autoantigen and the magnitude of EAMG**

<table>
<thead>
<tr>
<th>Groups of Mice</th>
<th>No. of Mice</th>
<th>Treatment</th>
<th>T Cell Response to TACHr Day 5</th>
<th>T Cell Response to TACHr Day 75</th>
<th>Clinical Score ± SD</th>
<th>Disease Incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>5</td>
<td>Control IgG2a</td>
<td>3536 ± 623</td>
<td>3120 ± 232</td>
<td>2.6 ± 0.3</td>
<td>71</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>5</td>
<td>Anti-CD25</td>
<td>3824 ± 420</td>
<td>3577 ± 412</td>
<td>2.8 ± 0.5</td>
<td>75</td>
</tr>
</tbody>
</table>

* Mice were immunized with TACHr/CFA and treated with α-GalCer, as described in Materials and Methods. Groups of mice were treated with control Abs or anti-CD25 mAb at day 0, and treatment of these Abs was repeated every 5 days until the termination of experiments. EAMG was monitored for up to 75 days p.i. TACHr-induced T cell proliferation was measured at days 5 and 75 p.i.
of IL-2 in supporting Treg cells. Early intervention in the pathogenetic process and introduction of additional regulatory compounds (e.g., copolymer) should achieve better therapeutic efficacy.

Based on the present study, we propose that cytokines and chemokines produced by locally activated NKT cells may promote the generation, recruitment, and maintenance of Treg cells to target organs. Subsequently, Treg cells can amplify the therapeutic effect of α-GalCer/NKT cells. Because α-GalCer stimulates both murine and human NKT cells (2), our results have direct implications for human disease. If confirmed in additional experimental systems, our findings will not only contribute to the understanding of the human disease. If confirmed in additional experimental systems, and human NKT cells (2), our results have direct implications for autoimmune myasthenia gravis: identification of regulatory cells.

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Disclosures

The authors have no financial conflict of interest.

References


Table III. CD4+CD25+ Treg cell and IL-2 are crucial for the therapeutic effects of α-GalCer

<table>
<thead>
<tr>
<th>Groups of Mice</th>
<th>No. of Mice</th>
<th>Treatment</th>
<th>Onset of Disease (day)</th>
<th>Clinical Score/Incidence</th>
<th>IgG (OD 405)</th>
<th>Cell Proliferation (cpm)</th>
<th>IFN-γ (pg/ml)</th>
<th>IL-4 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>8</td>
<td>Vehicle</td>
<td>21.0 ± 2.2</td>
<td>2.5 ± 0.4 (72%)</td>
<td>1.72 ± 0.2</td>
<td>2672 ± 334</td>
<td>303 ± 67</td>
<td>110 ± 19</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>9</td>
<td>α-GalCer, control Ab</td>
<td>33.1 ± 5.2</td>
<td>1.1 ± 0.7 (25%)</td>
<td>0.92 ± 0.2**</td>
<td>186 ± 27**</td>
<td>178 ± 23**</td>
<td>132 ± 21</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>12</td>
<td>α-GalCer, anti-CD25</td>
<td>19.7 ± 3.8</td>
<td>2.8 ± 0.3 (84%)</td>
<td>1.92 ± 0.3**</td>
<td>3102 ± 322**</td>
<td>387 ± 266**</td>
<td>144 ± 25</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>17</td>
<td>Vehicle</td>
<td>26.1 ± 2.4</td>
<td>2.4 ± 0.5 (74%)</td>
<td>1.83 ± 0.3</td>
<td>2356 ± 178</td>
<td>326 ± 55</td>
<td>97 ± 12</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>7</td>
<td>α-GalCer, control Ab</td>
<td>34.0 ± 2.1</td>
<td>1.2 ± 0.6 (22%)</td>
<td>0.78 ± 0.2**</td>
<td>1011 ± 250*</td>
<td>182 ± 12*</td>
<td>114 ± 23</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>8</td>
<td>α-GalCer, anti-IL-2</td>
<td>20.2 ± 3.8</td>
<td>2.5 ± 0.7 (81%)</td>
<td>1.34 ± 0.4**</td>
<td>2231 ± 312*</td>
<td>245 ± 31*</td>
<td>82 ± 15</td>
</tr>
</tbody>
</table>

* Mice were immunized with TACH/RCA and treated with α-GalCer, as described in Materials and Methods. Groups of mice were treated with control Abs or anti-CD25 mAb and anti-IL-2 mAb at day 0, and treatment of these Abs was repeated every 5 days until the termination of experiments. EAMG was monitored for up to 75 days p.i. AChR-induced T cell proliferation, and production of IFN-γ, IL-4, and anti-AChR IgG were measured at day 75 p.i. Statistical analysis was performed between control and corresponding experimental groups. *, p < 0.05; **, p < 0.01.