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Expansion of regulatory T cells *via* IL-2/anti-IL-2 mAb complexes suppresses experimental myasthenia

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Abstract

Human autoimmune diseases are often characterized by a relative deficiency in CD4⁺CD25⁺ regulatory T cells (Treg). We therefore hypothesized that expansion of Treg can ameliorate autoimmune pathology. We tested this hypothesis in an experimental model for autoimmune myasthenia gravis (MG), a B-cell-mediated disease characterized by auto-Ab directed against the acetylcholine receptor within neuromuscular junctions. We showed that injection of immune complexes composed of the cytokine IL-2 and anti-IL-2 mAb (JES6-1A12) induced an effective and sustained expansion of Treg, *via* peripheral proliferation of CD4⁺CD25⁺Foxp3⁺ cells and peripheral conversion of CD4⁺CD25⁺Foxp3⁺ cells. The expanded Treg potently suppressed autoreactive T- and B-cell responses to acetylcholine receptor and attenuated the muscular weakness that is characteristic of MG. Thus, IL-2/anti-IL-2 mAb complexes can expand functional Treg *in vivo*, providing a potential clinical application of this modality for treatment of MG and other autoimmune disorders.

Keywords

Autoimmunity; IL-2/IL-2 mAb complexes; Myasthenia Gravis; Self-tolerance; Treg

Introduction

Immune tolerance to self is maintained by multiple mechanisms and at multiple levels. A key mechanism of the immune system to prevent autoimmune reactivity is through the suppressive activity of CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Treg) [1–3]. In most autoimmune diseases, either systemic or organ-specific, reduced numbers and/or functions

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of Treg are often observed [4]. Myasthenia gravis (MG) is one of the best characterized human autoimmune disorders. In over 80% of these patients, aberrant Ab are generated against the acetylcholine receptors (AChR) at the neuromuscular junction. As a consequence, the neuromuscular transmission is impaired and patients suffer from various degrees of muscular weakness. Despite significant progress in the understanding of cognate interactions between APC, T cells and B cells in the production of auto-Ab and an increased understanding of the immune effector functions of auto-Ab, little is known regarding the mechanisms responsible for the breakdown of tolerance against AChR in MG. In keeping with many other types of autoimmune diseases, recent studies have suggested that MG patients have defects in Treg. Compared with healthy controls, numbers of circulating CD4⁺CD25^{high} Treg were decreased in some studies, but normal in other studies [5–10]. Elegant work by Balandina and colleagues documented that thymic Treg, although present in normal numbers, were severely impaired in their suppressor functions [5].

One question that remains unresolved is whether the deficit of Treg in MG patients is primary or secondary to the autoimmune pathology. Furthermore, it is not known whether restoration of Treg can re-establish immune tolerance to AChR in MG. To address these issues, we employed an animal model of MG, experimental autoimmune myasthenia gravis (EAMG) in mice. EAMG can be induced in C57BL/6 (B6) mice with purified AChR and adjuvant, which activates T cells and initiates production of anti-AChR Ab. Immunized mice exhibit chronic muscular weakness and electrophysiological features characteristic of MG. In this model, we previously demonstrated that IL-2 produced by activated NKT cells was able to expand Treg and improve the clinical outcome of EAMG [11]. Further, another study has shown that adoptive transfer of *ex vivo* expanded Treg can suppress EAMG in a rat model [12].

Here, we employed immune complexes consisting of IL-2 and anti-IL-2 mAb (JES6-1A12) (referred to as IL-2 complexes hereafter) to expand Treg. Consistent with earlier reports in other model systems [13–20], we found that anti-IL-2 mAb engaged CD25 (IL-2R α) in the high-affinity IL-2 receptor (IL-2R α , β , γ c), which induced a three- to four-fold expansion of Treg in the EAMG model. We also report the mechanism of Treg expansion in our model, dissect its impact on autoreactive T- and B-cell responses, and discuss the prospects and challenges for using this approach to treat MG and other autoimmune diseases.

Results

IL-2 complexes effectively expand Treg with stable Foxp3 expression in EAMG

Treg are essential for the maintenance of peripheral tolerance and prevention of autoimmune diseases [21]. A decreased population or functional impairment of these cells in MG patients and EAMG in rats [5, 12, 22] has been reported. To investigate the capacity of IL-2 complexes to expand Treg during EAMG in B6 mice and to address whether these expanded Treg were maintained during the course of EAMG, we first performed an experiment to determine the optimal regimen to administer IL-2 complexes. We found that a treatment protocol of two injections *per* week was optimal for initiating and maintaining the expansion of Treg *in vivo* (Supporting Information Table 1). We measured the percentages and numbers of Treg among splenic lymphocytes in mice treated with IL-2 complexes during EAMG. As shown in Fig. 1A–C, the percentages and numbers of CD4⁺ CD25^{high} Treg were consistently increased 4.4- to 8.7-fold in the IL-2 complex-treated mice as compared with isotype-treated control mice during the course of EAMG, and especially at the peak stage of disease (9.6% in IL-2 complex-treated mice *versus* 1.1% in isotype-treated mice on day 35 post-immunization (p.i.), $p < 0.001$). Similar results were obtained when lymphocytes from lymph nodes and peripheral blood were analyzed (data not shown).

Foxp3 is a transcription factor that plays a critical role in the development and functional maturation of the Treg lineage [23, 24]. Our finding that the percentage and absolute numbers of CD4⁺CD25^{high} cells in mice treated with IL-2 complexes are profoundly increased led us to evaluate Foxp3 expression in the expanded cells. The majority of CD4⁺CD25^{high} cells in both control mice and mice treated with IL-2 complexes expressed Foxp3, suggesting that the effect of IL-2 complexes on Treg was not qualitative but quantitative (Fig. 1D–F). The finding that the absolute numbers of Treg in the animals treated with IL-2 complexes were increased (Fig. 1E) further supported this conclusion. At the peak of disease at day 35 p.i., numbers of Treg in AChR-immunized mice treated with IL-2 complexes were increased 13.3-fold as compared with AChR-immunized mice treated with isotype control Ab and were increased 5.4-fold as compared with naïve mice. Therefore, we concluded that IL-2 complexes induced CD4⁺CD25^{high} cells with stable expression of Foxp3. Similar results were obtained when lymphocytes from lymph nodes or peripheral blood were analyzed (data not shown).

IL-2 complexes failed to induce significant alterations in other white blood cells, including CD4⁺ T, CD8⁺ T, CD11b⁺, CD11c⁺, NK and NKT cells (Supporting Information Fig. 1).

Effects of IL-2 complexes on the homeostasis of Treg in Foxp3^{gfp} mice

We used Foxp3^{gfp} mice [23] to provide further support for our findings, and to compare the efficacy of IL-2 complexes, IL-2 alone, and anti-IL-2 mAb alone in expanding Treg. We found that the frequency of CD4⁺CD25^{high} Treg in the draining lymph nodes of AChR-primed Foxp3^{gfp} mice treated with IL-2 complexes was increased 3.5- to 5.1-fold when compared with mice treated with isotype control Ab, IL-2 or anti-IL-2 mAb alone (Fig. 2A). Similar results were obtained for CD4⁺Foxp3⁺ cells (Fig. 2B) and CD25⁺ Foxp3⁺ cells (Fig. 2C). Foxp3 was expressed in the majority of CD4⁺CD25^{high} cells (isotype control IgG: 91±17%, IL-2: 93±3%, anti-IL-2 mAb: 85±7% and IL-2 complexes: 92±5%) and the levels of Foxp3 expression by Treg among the different groups were not statistically different. Similar findings were obtained in the spleen and peripheral blood (data not shown). These results confirm our findings presented in Fig. 1 and further indicate that IL-2 complexes are more efficient than IL-2 alone in expanding Treg. Additional experiments indicated that TGF-β, which is critical for Treg development, did not synergize with IL-2 complexes for Treg expansion but slightly increased the levels of Foxp3 expression (Supporting Information Fig. 2A, B and D).

IL-2 complexes expand Treg *via* proliferation and conversion of CD4⁺CD25⁺ and CD4⁺CD25[−] cells, respectively

Naturally occurring CD4⁺CD25⁺ Treg are derived from the thymus as a functionally mature T-cell subpopulation and then distribute into the periphery [25]. CD4⁺CD25⁺ Treg can also be induced in the periphery or in *ex vivo* cultures from CD4⁺CD25[−] T cells using TGF-β [26]. In the present study, CD4⁺CD25^{high}Foxp3⁺ cells expanding in response to IL-2 complexes were found in the peripheral lymphoid organs (Fig. 1 and 2) as well as in the circulation (data not shown). To determine whether these expanded Treg were generated in the periphery or in the thymus, we examined the population of CD4⁺CD25⁺ cells in the thymus of Foxp3^{gfp} mice immunized with AChR. The frequencies of Treg as well as their expression of Foxp3 in the thymus were comparable in control mice and in mice receiving IL-2 complexes (Fig. 3A–C). Furthermore, in *ex vivo* experiments of CD4⁺CD25⁺Foxp3^{gfp} cells (CD4⁺CD25⁺Foxp3⁺ cells 4.95%) and CD4⁺CD25[−]Foxp3^{gfp} cells (CD4⁺CD25⁺Foxp3⁺ cells <0.5%) cultured in the presence of IL-2 complexes, we found that cells from CD4⁺CD25⁺Foxp3⁺ cell cultures (Fig. 3D, 8.3% BrdU-labeled CD4⁺ cells after culture) proliferated more extensively than cells from CD4⁺CD25[−]Foxp3[−] cell cultures (Fig. 3D, 3.5% BrdU-labeled CD4⁺ cells after culture). The frequency of CD4⁺CD25⁺Foxp3^{gfp}+

cells from the proliferating cells in the CD4⁺CD25⁻Foxp3^{gfp}- cell cultures (identified by BrdU labeling) was significantly increased (Fig. 3F; % of CD4⁺CD25⁺ cells: <0.05 *versus* 6.7, before *versus* after culture, respectively). As expected, no changes were observed in the CD4⁺CD25⁺Foxp3^{gfp}+ cell cultures (Fig. 3F; % of CD4⁺CD25⁺ cells: >95 *versus* 96.4, before *versus* after culture, respectively). The absolute number of CD4⁺CD25⁺Foxp3^{gfp}+ cells from the proliferating cells in the CD4⁺CD25⁺Foxp3^{gfp}+ cell cultures (30 285 *cells per* 10⁶ cultured cells) was 8.7-fold higher than that in the CD4⁺CD25⁻Foxp3^{gfp}- cell cultures (3492 *cells per* 10⁶ cultured cells). However, *in vivo*, the number of CD4⁺CD25⁻Foxp3⁻ T cells far exceeded (more than seven-fold) the number of CD4⁺CD25⁺Foxp3⁺ T cells (*i.e.* B6 or Foxp3^{gfp} mouse lymphocytes: ~15% CD4⁺CD25⁻Foxp3⁻ T cells *versus* <2% CD4⁺CD25⁺Foxp3⁺ T cells). Thus, CD4⁺CD25⁺ Foxp3⁺ and CD4⁺CD25⁻Foxp3⁻ T cells both might provide a source of Treg expansion. In addition, we demonstrated that IL-2 complexes can also expand Foxp3^{gfp}+ Treg in thymectomized Foxp3^{gfp} mice (Fig. 4). These data suggested that the proliferation of Treg themselves, together with conversion of CD4⁺CD25⁻Foxp3⁻ cells into Treg outside of the thymus, is a major means by which IL-2 complexes expand Treg. However, the contribution of thymic Treg cannot be fully excluded.

Treg expanded by IL-2 complexes during EAMG have immunosuppressive activities

Having determined the effects of IL-2 complexes on the expansion and maintenance of Treg, we investigated next whether the expanded CD4⁺CD25^{high}Foxp3⁺ Treg exhibited a suppressive function, similar to naturally occurring Treg. Thus, we compared the capacity of Treg from control mice and mice receiving IL-2 complexes to suppress the proliferation of effector T cells. For this purpose, freshly isolated CD4⁺CD25⁻ responder cells from AChR-primed mice treated with isotype control Ab were mixed at a ratio of 1 to 1 with CD4⁺CD25⁺ T cells purified from AChR-primed mice treated with either IL-2 complexes or isotype control Ab. The CD4⁺CD25⁺ Treg from IL-2 complex-treated and isotype control IgG-treated mice suppressed the proliferation of the responder cells at a similar magnitude (% of inhibition of proliferation: 22±11 and 34±9%, respectively). Figure 5 shows that at Treg:responder cell ratios of 1:1 to 1:20 (CD4⁺CD25⁺ cells: CD4⁺CD25⁻ cells), the activities of Treg from IL-2 complex- and isotype control-treated mice suppressed T-cell proliferation stimulated with AChR, anti-CD3, and Con A to a similar degree. Thus, our results suggested that Treg expanded by IL-2 complexes are as potent as naturally occurring Treg in suppressing the proliferation of responder T cells.

IL-2 complexes ameliorate EAMG

To test the influence of IL-2 complexes on the course of EAMG, we treated mice with IL-2 complexes at the time of disease induction and monitored these animals for clinical signs of MG for 12 wk after AChR immunization. Control B6 mice developed moderate to severe clinical muscle weakness, whereas the mice receiving IL-2 complexes exhibited relatively mild disease symptoms. Although the onset of EAMG in both groups was similar, between weeks 3 and 5 p.i., the cumulative disease incidence was significantly higher in control B6 mice (77.8±15.7%) than in IL-2 complex-treated mice (42.8±11.8%). On week 5 p.i. after booster immunizations, the clinical score of control mice was maximal at 1.81±0.43. By contrast, even after booster immunizations, the maximal clinical score of mice treated with IL-2 complexes was 0.63±0.18 ($p<0.01$) (Fig. 6A; Supporting Information Table 2). IL-2 complexes also effective when administered at a time when signs of muscular weakness were already apparent (Fig. 6B). Transfer of Treg isolated from IL-2 complex-treated mice also reduced the severity of MG in recipient mice (Fig. 6C). Therefore, IL-2 complexes are an effective treatment to ameliorate EAMG.

IL-2 complexes decrease AChR-reactive T-cell responses

In murine EAMG, it has been shown that AChR-specific CD4⁺ T cells play a critical role in helping B cells to produce pathogenic anti-AChR Ab [27]. Treg play a role in suppressing CD4⁺ T-cell responses and production of characteristic Th1, Th2 and Th17 cytokines [28]. We employed a proliferation assay to determine autoreactive T-cell responses in EAMG. Compared with control mice, T cells from mice treated with IL-2 complexes exhibited significantly reduced levels of proliferative responses to AChR (Fig. 7A). However, there was no difference in the response to Con A-induced T-cell proliferation between these two groups (Fig. 7B).

Next, we measured cytokine production. Compared with B6 mice treated with isotype controls, mice treated with IL-2 complexes had markedly suppressed IFN- γ responses to stimulation with AChR (Fig. 7C); however, we did not detect any significant differences in IL-17-producing T cells (Fig. 7C). Regarding Th2 cell responses, the levels of IL-4 in mice treated with IL-2 complexes were significantly increased as compared with mice treated with isotype control Ab (Fig. 7D). IL-10 was undetectable in all cultures both by ELISA and flow cytometry. These data suggested that administration of IL-2 complexes skews T-cell responses toward Th2 cell differentiation.

A reciprocal relationship between Th17 cells and Foxp3⁺ Treg has been proposed [29]. As the development of both Th17 cells and Treg involves TGF- β , addition of IL-6 would be expected to preferentially skew the response toward Th17 differentiation. To investigate the expression of cytokines that critically influence commitment toward the Treg and Th17 cell lineages, we compared the levels of TGF- β and IL-6. Strikingly, levels of TGF- β were significantly increased in mice treated with IL-2 complexes (Fig. 7E), whereas the levels of IL-6 were similar in the two groups (Fig. 7F).

Effects of IL-2 complexes on autoreactive B-cell responses

EAMG is mainly mediated by IgG Ab to AChR and these Ab are the primary effectors for disease development. The effects of IL-2 complexes treatment on serum anti-AChR Ab responses were determined by ELISA. Compared with mice treated with isotype control Ab, mice treated with IL-2 complexes had a significant reduction in anti-AChR IgG2b ($p < 0.01$) and IgG3 ($p < 0.005$), which was accompanied by increased levels of anti-AChR IgG1 ($p < 0.01$) and IgG2a ($p < 0.05$). The total amounts of anti-AChR IgG were not significantly altered (Fig. 8A and B). This profile of anti-AChR IgG subclasses might contribute to reduced levels of EAMG mediated by treatment with IL-2 complexes. We also examined the frequency of B cells and plasma cell differentiation in mice receiving IL-2 complexes. Compared with control mice, the frequency of CD19⁺ B cells was significantly reduced (Fig. 8C), the frequency of IgD⁺IgM⁺ B cells had a tendency for reduced levels (Fig. 8D), but the differentiation of plasma cells did not appear to be affected (Fig. 8E).

Discussion

Treg play a nonredundant role in maintaining immunological self-tolerance and immune homeostasis. The importance of these cells is made evident by the observation that depletion of Treg from normal rodents produces a variety of autoimmune inflammatory disorders, whereas reconstitution with Treg can inhibit autoimmune disease development [3, 30, 31]. Interestingly, a characteristic immunological feature in patients with autoimmune diseases, including MG, is that Treg are present in reduced numbers and/or have compromised functions, suggesting the possibility that human autoimmune pathology is associated with defective Treg. In such a scenario, restoration or expansion of Treg should ameliorate

autoimmune-mediated tissue destruction. Our study has addressed this hypothesis in EAMG, a T-cell-dependent, Ab-mediated autoimmune disease of neuromuscular junctions.

The common γ -chain cytokine IL-2 plays a critical role in regulating the homeostasis of Treg [32]. To expand Treg, we took advantage of IL-2/anti-IL-2 mAb immune complexes that were initially described by Boyman *et al.* [13] and Kamimura *et al.* [16]. The mechanism by which IL-2 complexes exhibit superior capacity to treatment with IL-2 or anti-IL-2 mAb alone in expanding Treg has been previously studied. It has been suggested that immune complexes containing anti-IL-2 mAb increase the half life of IL-2 *in vivo* [33, 34]. Nevertheless, the precise mechanisms remain elusive.

In addition to demonstrating a selective and efficient expansion of Treg, our studies have revealed a number of new aspects of treatment with IL-2 immune complexes. First, we have developed a regimen for long term administration (up to 12 wk). This regimen induced a four- to eight-fold increase in Treg in spleen, lymph nodes and circulating blood. Similar to naturally occurring Treg, the expanded Treg acquired stable Foxp3 expression. This regimen did not significantly alter other lymphocyte populations, except for B cells. Second, our findings indicated that the expanded Treg were derived from peripheral expansion of CD4⁺CD25⁺Foxp3⁺ cells and peripheral conversion of CD4⁺CD25⁻ cells, as the frequency and number of Treg in the thymus remained unaltered by IL-2 complexes. Most importantly, the expanded Treg were capable of inhibiting responder CD4⁺ T-cell proliferation in an antigen-specific or antigen-independent fashion. The capacity of the expanded cells to suppress effector T-cell responses was similar to that of naturally occurring Treg cells. Finally, the expansion of Treg was associated with a reduction in the incidence and a milder muscular weakness in EAMG. We also observed a dramatic reduction in Th1 cell responses, a concurrent increase in Th2 cell responses, and a corresponding shift in IgG isotypes directed against AChR.

Consistently, levels of TGF- β production were augmented in the AChR-primed mice that were treated with IL-2 complexes. TGF- β , in conjunction with IL-6, also promotes the differentiation of Th17 cells [29, 35]. However, we failed to observe an increase in IL-17 responses in these mice. The cellular source of TGF- β as well as the contribution of TGF- β to Treg expansion is currently under investigation.

Suppression of Th cells and/or Ab-producing B cells would be expected to improve clinical outcomes of EAMG. Clearly, AChR-reactive Th cells with a Th1 phenotype were suppressed, whereas Th2 phenotype cells were augmented in mice that received IL-2 complexes. This skewing of immune responses might contribute to the capacity of IL-2 complexes to suppress disease. On the other hand, the frequency of B cells in mice that receiving IL-2 complexes was reduced, whereas plasma cell differentiation was not significantly impaired. Whether the effects of IL-2 complexes on B cells are attributed to impaired Th cell responses or directly mediated by the immune complexes is unclear.

During the assembly of this manuscript, a report by Webster *et al.* provided evidence that IL-2 complexes can protect mice against experimental autoimmune encephalomyelitis and against rejection of pancreatic islet allografts [14]. Here, we have expanded the therapeutic capacity of IL-2 complexes to the Ab-mediated disease EAMG. Consistent with the paper by Webster *et al.* [14], we have demonstrated that IL-2 complexes can induce a sustained expansion of Treg with stable expression of Foxp3 and robust suppressive capacity. Collectively, these studies provide a strong rationale to explore the therapeutic properties of IL-2 complexes in autoimmune and inflammatory diseases.

Materials and methods

Mice

We purchased female B6 mice, 7–8 wk of age from Taconic Labs (Germantown, NY, USA). Foxp3^{gfp} mice [23] on the B6 background were originally from Alexander Y. Rudensky (University of Washington, Seattle, WA, USA) and these mice express a GFP “knocked in” a Foxp3 allele so that the expression of Foxp3 can be detected by FACS staining. All mice were housed and handled in pathogen-free conditions according to approved protocols of the Institutional Animal Care and Use Committee at the Barrow Neurological Institute, St. Joseph’s Hospital and Medical Center.

Antigens and peptides

Torpedo AChR (tAChR) was purified from *Torpedo californica* electric organ (Pacific Biomarine, Venice, CA, USA) by affinity chromatography using neurotoxin α -cobratoxin (Sigma, St. Louis, MO, USA) coupled to Sepharose 4 Fast Flow agarose (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) [11, 36]. Mouse AChR was purified from mouse muscle tissues as previously described [11, 36]. The purified receptor was analyzed on SDS-PAGE. Synthetic murine AChR $\alpha_{146-162}$ peptide (L-G-I-W-T-Y-D-G-T-K-V-S-I-S-P-E-S) was purchased from Biosynthesis (Lewisville, TX, USA).

Induction and clinical evaluation of EAMG

Female B6 mice were immunized with 20 μ g of tAChR emulsified in CFA (DIFCO, Lawrence, KS, USA), by s.c. injection of 200 μ L of suspension in the shoulder and tail base, and boosted with 20 μ g of tAChR in incomplete Freund’s adjuvant (IFA) every 30 days p.i. For clinical examination, mice were evaluated and scored every other day for myasthenic muscle weakness and assigned clinical scores as previously described [11]. Briefly, mice were observed on a flat platform for a total of 3 min. Then, mice were exercised by repetitive paw grips on a cage top grid for 1 min and followed by another 3min observation for signs of EAMG. Disease severity of muscle weakness was expressed as follows: grade 0, normal posture, muscle strength and mobility; grade 1, normal at rest but with muscle weakness shown by a hunchback posture, difficulty in raising the head, and mildly decreased activity after exercise; grade 2, as grade 1 without exercise; grade 3, as grade 2 weakness with moribund, dehydrated and paralyzed; and grade 4, dead [11]. Clinical EAMG in sick mice was confirmed by neostigmine bromide/atropine sulfate test.

Preparation and administration of IL-2 complexes

Carrier-free recombinant mouse IL-2 and functional grade purified anti-mouse IL-2 mAb (JES6-1A12) were obtained from eBioscience (San Diego, CA, USA). IL-2 and anti-IL-2 mAb complexes (referred to as IL-2 complexes) were prepared as previously described [13, 16]. In brief, equal volumes of 30 μ g/mL rIL-2 and 1 mg/mL anti-mouse IL-2 mAb in PBS were mixed *in vitro* and 200 μ L of this solution was injected i.p. into each mouse, resulting in IL-2 complexes formed by 1.5 mg of rIL-2 with 50 mg of IL-2 mAb per mouse. As controls, animals were treated with rIL-2 or IL-2 mAb only, and received 200 μ L of PBS with the same amount of either rIL-2 or IL-2 mAb as for the IL-2 complexes. Additional control mice received equivalent doses of purified normal rat isotype control IgG (Sigma-Aldrich). Injections was repeated two times every week starting on the same day of AChR immunization (day 0) or 35 days p.i. until termination of the experiment.

Thymectomy

Seven- to eight-week-old Foxp3^{gfp} mice were weighed and anesthetized with ketamine (80 mg/kg) and xylazine (12 mg/kg) by i.p. injection, and the thymus was surgically removed. The wound were sutured and the mice received Ab treatment 1 wk after thymectomy.

Adoptive transfer

To test the *in vivo* regulatory function of Treg expanded by IL-2 complexes, we immunized Foxp3^{gfp} mice with AChR/CFA as described above. On day 20 after immunization, we isolated the Foxp3^{gfp} cells from Foxp3^{gfp} mice treated with isotype control IgG or IL-2 complexes for 7 consecutive days (one dose/day). The purity of isolated Foxp3^{gfp} cells was verified as > 95% by flow cytometry. For adoptive transfer experiments, 1×10⁶ purified Foxp3^{gfp} cells treated with control IgG or IL-2 complexes were immediately injected i.v. into each AChR-immunized recipient mouse (on day 15 p.i.) and then their clinical EAMG presentation of muscle weakness was observed.

Lymphocyte isolation and cell proliferation assay

Single mononuclear cell (MNC) suspensions of spleens, draining lymph nodes or thymuses from mice were prepared as previously described [11]. Cells (4×10⁵ MNC/200 µL medium/well) were cultured for 72 h in the presence of AChR (10 µg/mL), AChR_{146–162} (10 µg/mL), or Con A (5 µg/mL), or with the indicated Ab. The rate of cell proliferation was determined by adding 1 µCi [³H]-thymidine/well (specific activity of 42 Ci/mmol; Amersham, Arlington Heights, IL, USA) for the last 18 h, followed by liquid scintillation counting using a Wallac MicroBeta Counter (PerkinElmer, Waltham, MA, USA). Results were expressed as mean cpm (counts *per minute*) ± SEM of triplicate wells [11].

Treg in vitro suppression assays

MNC were obtained from AChR-primed mouse spleen and CD4⁺ CD25⁺ or CD4⁺CD25[−] T cells were purified by a mouse CD4⁺ CD25⁺ Regulatory T cell Isolation Kit^{TM} (Miltenyi Biotec, Auburn, CA, USA) as previously described [11]. Purities of isolated T cells were verified as 95% by flow cytometry.

Various numbers of CD4⁺CD25⁺ T cells (5~50×10³) were cultured with 50×10³ CD4⁺CD25[−] T cells (as responders) and 50×10³ irradiated splenocytes (2000 rad) in a total volume of 200 µL media in the presence of AChR (10 µg/mL), anti-CD3 (0.2 µg/mL, clone 145.2C11) or Con A (1 µg/mL) for 72 h and pulsed with 1 mCi/well of [³H]-thymidine for the last 18 h of the culture. Cell proliferation responses were assessed by measuring [³H]-thymidine incorporation as the mean cpm ± SEM of triplicate cultures [37].

In vitro cultures of CD4⁺CD25⁺Foxp3^{gfp} and CD4⁺ CD25[−]Foxp3^{gfp} cells

Spleen MNC were obtained from Foxp3^{gfp} mice, labeled with anti-CD4-PE and anti-CD25-allophycocyanin, and sorted into CD4⁺CD25⁺ Foxp3^{gfp} or CD4⁺CD25[−]Foxp3^{gfp} T cells (purities 95%) by using a FACSaria flow cytometer (Becton Dickinson, Mountain View, MD, USA). 1×10⁶ cells/mL/well of CD4⁺CD25⁺ Foxp3^{gfp} or CD4⁺CD25[−]Foxp3^{gfp} T cells were cultured with IL-2 (150 ng/mL), anti-IL-2 mAb (5 µg/mL), IL-2 complexes (150 ng/mL of IL-2+5 µg/mL of IL-2 mAb), TGF-β (5 ng/mL), or anti-TGF-β mAb (5 µg/mL) for 4 days at 37°C and then stained with anti-CD4 and anti-CD25 Ab.

Flow cytometric analysis

Flow cytometry was performed as previously described [38]. Briefly, for surface staining, 10⁶ cells of spleens, lymph nodes or thymuses from naïve or AChR-immunized mice with or without the indicated treatment were incubated with the following primary mAb (BD

Pharmingen, San Diego, CA, USA): CD3 (145–2C11), CD4 (GK1.4), CD8 (53–6.7), CD11b (M1/70), CD11c (HL3), CD19 (1D3), CD25 (7D4), CD138 (281–2), and NK1.1 (PK136), and conjugated with one of the following fluorescent tags: FITC, PE, PerCP, allophycocyanin, PC5 or PC7. For intracellular cytokine staining, 2×10^6 cells/mL were cultured in 24-well plates with or without antigens (10 μ g/mL AChR, 10 μ g/mL AChR $_{\alpha 146-162}$, or 2 μ g/mL Con A) for 4 days and then stimulated with 20 ng/mL PMA and 1 μ g/mL ionomycin (Sigma-Aldrich) in the presence of GolgiPlug (BD Bioscience) for 5 h prior to staining. Cells were surface-stained with anti-CD4- FITC, fixed and permeabilized with Cytofix/Cytoperm kit (BD Bioscience), and then intracellular cytokines were stained with anti-IFN- γ , anti-IL-4, and anti-IL-17 mAb conjugated with Alexa 647 or PE. For intracellular Foxp3 expression, 10^6 cells were stained for surface molecules with anti-CD4-FITC (H129.19) and anti-CD25-allophycocyanin (PC61.5), fixed and permeabilized, and then stained for intracellular molecules with anti-Foxp3-PE (FJK-16s) (eBioscience). Samples were read using a FACS Aria^{TM} flow cytometer and data analyzed using Diva^{TM} software.

BrdU incorporation assay *in vitro*

To label the cultured cells, 10 μ L of 1mM BrdU was added to 1mL of culture media. After 16–18 h, BrdU-labeled cells were harvested and surface stained with PE-Cy7-labeled anti-CD4, PE-labeled anti-CD25 and allophycocyanin-labeled anti-BrdU mAb (BrdU Flow Kit, BD Biosciences), followed by flow cytometry [38].

Measurement of anti-AChR Ab

Sera of treated mice were collected around p.i. week 6 (day 35~40). Anti-murine AChR IgG Ab were detected by ELISA as follows [11, 36]. Microtiter plates were coated with murine AChR (0.5 μ g/mL) and reacted with 100 μ L of mouse sera at optimal dilution factor, which for each Ab was within the linear range of the assay (1/400 for total IgG, IgG1, IgG3 and IgM; 1/200 for IgG2b; 1/40 for IgG2a). HRP-conjugated rabbit anti-mouse IgG, IgG1, IgG2a, IgG2b, IgG3, or IgM (1/1000, Invitrogen, Carlsbad, CA, USA) were added and followed by color development with TMB (3, 3', 5, 5'-tetramethylbenzidine) substrate. Ab levels were evaluated by measuring OD at 450 nm.

RIA for serum anti-AChR Ab concentration

Serum samples were collected at 6 wks (day 35~40) p.i. from B6 mice treated with isotype control mAb or IL-2 complexes and serum Ab concentrations to mAChR were measured by RIA as previously described [39]. Briefly, 1 nmol mouse AChR was incubated with 2 nmol ^{125} I- α -bungarotoxin (BGT) (Amersham). To 1mL of labeled mAChR, 1 μ L serum was added, followed by rabbit anti-mouse Ig. The samples were centrifuged, washed and counted in a gamma counter. The AChR precipitate minus the background value permits calculation of the titer in moles of toxin-binding sites bound *per* liter of serum.

Cytokine ELISA assay

Standard ELISA for detection of mouse serum IL-2, IL-4, IL-6, IL-10 and TGF- β 1 were performed using BD OptEIA ELISA kit (BD Biosciences Pharmingen) as previously described [38]. Serum samples were collected at the indicated p.i. time points from AChR-immunized mice treated with or without IL-2 complexes. Results were expressed as mean cytokine concentration (pg/mL) $\pm$ SEM.

Statistics

Results were presented as means $\pm$ SEM, and analysis was performed with the aid of GraphPad Prism software (San Diego, CA, USA). Nonparametric Mann–Whitney *U* test

was utilized for evaluation of clinical scores. One-tailed unpaired Student's *t*-test or ANOVA was used as appropriate for all other comparisons among groups (Student's *t*-test: two groups; ANOVA: multiple groups). Significance was defined as $p < 0.05$ and expressed in the individual figures as * $p < 0.05$, ** $p < 0.01$; NS: not statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AChR	acetylcholine receptor
B6	C57BL/6 mice
EAMG	experimental autoimmune myasthenia gravis
MG	myasthenia gravis
MNC	mononuclear cell
p.i.	post-immunization
tAChR	<i>Torpedo</i> AChR

Figure 1. Homeostasis of CD4⁺CD25^{high} Treg in AChR-primed mice treated with IL-2 complexes. Splenocytes from AChR-immunized B6 mice treated with isotype control IgG or IL-2 complexes were prepared on the indicated days after immunization, and stained with anti-CD4 and anti-CD25 mAb as described in the *Materials and methods* section. Dot plots were gated on lymphocytes. Results of frequencies of individual cell populations were pooled from three independent experiments ($n=2$ or 3 mice/group for each experiment). * $p<0.05$, ** $p<0.01$, ANOVA. (A) CD4⁺CD25^{high} Treg frequencies; (B) CD4⁺CD25^{high} Treg numbers; (C) kinetics of the CD4⁺CD25^{high} population during EAMG; (D) Foxp3⁺ Treg frequencies among CD4⁺CD25^{high} cells; (E) Foxp3⁺ Treg numbers among CD4⁺CD25^{high} cells; and (F) kinetics of the Foxp3⁺ cell population of CD4⁺CD25^{high} cells during EAMG.

Figure 2.

The profile of IL-2 complex-expanded CD4⁺CD25^{high} Treg in Foxp3^{gfp} mice. Lymphocytes from AChR-immunized Foxp3^{gfp} mouse draining lymph nodes treated with isotype control IgG, IL-2 alone, anti-IL-2 mAb alone or IL-2 complexes were prepared on day 35 after immunization and were stained with anti-CD4 and anti-CD25 mAb. Representative dot plots were gated on lymphocytes. Results of frequencies of individual cell populations were pooled from three independent experiments ($n=2$ or 3 mice/group for each experiment). * $p<0.05$, ** $p<0.01$; ANOVA. (A) CD4⁺CD25⁺ Treg; (B) CD4⁺Foxp3^{gfp}⁺ cells; and (C) CD25⁺Foxp3^{gfp}⁺ cells.

Figure 3.

IL-2 complexes expand Treg *via* proliferation of peripheral CD4⁺CD25⁺ T cells and conversion of extrathymic CD4⁺CD25⁻ T cells. On day 35 after immunization, thymocytes from the AChR-immunized Foxp3^{gfp} mice treated with isotype control IgG or IL-2 complexes were isolated from thymus by passing through a 40-μm cell strainer and stained with anti-CD4, and anti-CD25 Ab. Representative dot plots were gated on lymphocytes. Results of frequencies of individual cell populations were pooled from three independent experiments ($n=2$ or 3 mice/group for each experiment). (A) CD4⁺CD25⁺ Treg; (B) CD4⁺Foxp3^{gfp}⁺ cells; and (C) CD25⁺Foxp3^{gfp}⁺ cells. Splenocytes from Foxp3^{gfp} mice were separated into CD4⁺CD25⁺Foxp3^{gfp}⁺ or CD4⁺CD25⁻Foxp3^{gfp}⁻ T-cell subsets as described in the *Materials and methods*. CD4⁺CD25⁺Foxp3^{gfp}⁺ and CD4⁺CD25⁻Foxp3^{gfp}⁻ T cells (1×10^6 cells/mL) were cultured with IL-2 complexes for 4 days, and then harvested for staining with anti-CD4 and anti-CD25 Ab. Representative dot plots were gated on lymphocytes. Frequencies of the indicated cell populations are shown from one of three individual experiments with similar results. (D) Proliferation of T cells labeled *in vitro* with BrdU. (E) Bar plots of Foxp3⁺ cell frequencies and numbers. ** $p < 0.01$, Student's *t*-test. (F) Dot plots of the indicated T-cell frequencies.

Figure 4.

IL-2 complexes expand Treg in thymectomized Foxp3^{gfp} mice. Foxp3^{gfp} mice were thymectomized and then immunized with AChR/CFA, treated with isotype control IgG or IL-2 complexes. On day 35 after immunization, lymphocytes were prepared from these Foxp3^{gfp} mouse draining lymph nodes and stained with anti-CD4 and anti-CD25 mAb. Representative dot plots were gated on lymphocytes. Results of frequencies of individual cell populations were pooled from two independent experiments ($n=2$ or 3 mice/group for each experiment). * $p<0.05$, ** $p<0.01$; Student's t -test. (A) CD4⁺CD25⁺ Treg; (B) CD4⁺Foxp3^{gfp} cells; and (C) CD25⁺Foxp3^{gfp} cells. Data are summarized in (D).

Figure 5.

CD4⁺CD25^{high} Treg expanded by IL-2 complexes display suppressor activity. Purified CD4⁺CD25⁺ Treg and purified responder CD4⁺CD25⁻ T cells (1×10^5) from AChR-primed B6 mice treated with isotype control IgG or IL-2 complexes were prepared on day 35 after immunization and cultured at the indicated ratios and stimulated with (A) AChR (10 μ g/mL), (B) anti-CD3 (0.2 μ g/mL, clone 145.2C11), or (C) Con A (1 μ g/mL), in the presence of irradiated (2000 rad) splenocytes (50×10^3 cells/well) for 72h, and then pulsed with 1 μ Ci/well of [³H] thymidine for the last 18 h of the culture. Data are shown as mean [³H] thymidine incorporation in triplicate cultures. The data are representative of three separate experiments with similar outcomes. In control experiments, with responder cells only, levels

of proliferation were similar to those with Treg:T effector cell ratios of 0.05:1 (data not shown). ** $p < 0.05$, ** $p < 0.01$; Student's *t*-test.

Figure 6.

Impact of IL-2 complexes on EAMG. Groups of B6 mice treated with isotype control IgG or IL-2 complexes were immunized with AChR and monitored for clinical scores of muscle weakness as described in the *Materials and methods* section. (A) Treatment started on the same day as AChR immunization; (B) treatment started on day 35 after immunization (the arrows indicate the start of injection). Results are pooled from three independent experiments ($n=4-9$ mice/group in each experiment) and are expressed as daily mean clinical scores. Bars indicate standard errors. (C) Groups of B6 mice ($n=3-5$) received CD4⁺CD25⁺Foxp3⁺ cells from Foxp3^{gfp} mice treated with isotype control IgG or IL-2 complexes

(the arrow indicates the day of cell transfer). This experiment was repeated one time with similar results. * $p < 0.05$, ** $p < 0.01$; Mann–Whitney U test.

Figure 7.

Effect of IL-2 complexes on AChR-specific T-cell proliferation and cytokine production. Splenocytes from unmanipulated B6 mice or AChR-immunized B6 mice treated with or without IL-2 complexes were harvested on day 35~40 post-immunization or on the indicated days and analyzed for proliferative responses to (A) AChR (0.1, 1, 10 and 100 $\mu\text{g/mL}$) and (B) Con A (5 $\mu\text{g/mL}$) by [^3H] thymidine incorporation *in vitro*. The results are presented as cpm and are expressed as means $\pm$ SD (pooled from three independent experiments, $n=2$ or 3 mice/group for each experiment). Splenocytes from AChR-immunized B6 mice treated with or without IL-2 complexes were collected between weeks 5 and 6 p.i. and cultured in the presence of AChR (10 $\mu\text{g/mL}$) for 72 h. Intracellular cytokines were examined by flow

cytometry. Dot plots were gated on lymphocytes as shown in representative results from three separate experiments ($n=2$ or 3 mice/group in each experiment). The bars present percentages of the indicated cytokine-producing cells and are expressed as means $\pm$ SD. (C) AChR-induced IFN- γ and IL-17 production by CD4⁺ T cells. (D) AChR-induced IL-4 production by CD4⁺ T cells; each symbol represents one mouse. (E) Serum TGF- β profile. (F) Serum IL-6 profile. * $p<0.05$, ** $p<0.01$; Student's t -test.

Figure 8.

Effect of IL-2 complexes on humoral and AChR-reactive B-cell responses. (A) Serum samples were collected at 6 wk (day 35~40) post-immunization from B6 mice treated with isotype control mAb ($n=8$) or IL-2 complexes ($n=16$). Serum levels of anti-AChR-specific total IgG, IgG₁, IgG_{2a}, IgG_{2b}, IgG₃ and IgM were determined by ELISA on AChR-coated plates as described in the *Materials and methods* section. Serum dilution for total IgG, IgG₁, IgG₃ and IgM was 1/400, for IgG_{2b} 1/200, and for IgG_{2a} 1/40. The pooled data are expressed as mean OD values $\pm$ SE. (B) Serum anti-AChR Ab concentrations were measured by RIA and expressed as moles of toxin-binding sites bound *per* liter of serum ($n=4$ /group). Splenocytes from AChR-immunized mice treated with isotype control IgG or IL-2 complexes were prepared on day 35~40 after immunization, and stained with surface mAb. Dot plots were gated on lymphocytes in three independent experiments ($n=2$ or 3 mice/group *per* experiment); each symbol represents one mouse. (C) CD19⁺ cell frequency; (D) IgM⁺IgD⁺ cell frequency among CD19⁺ cells; (E) CD138⁺ cell frequency among CD19⁺ cells. The values between groups were compared; * $p<0.05$, $p<0.01$; NS indicates not statistically significant; Student's *t*-test.