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Ex Vivo Generated Regulatory T Cells Modulate Experimental Autoimmune Myasthenia Gravis\textsuperscript{1}

Revital Aricha,* Tali Feferman,* Sara Fuchs,\textsuperscript{2,*} and Miriam C. Souroujon*†

Naturally occurring CD4\textsuperscript{+}CD25\textsuperscript{+} regulatory T (Treg) cells are key players in immune tolerance and have therefore been suggested as potential therapeutic tools for autoimmune diseases. In myasthenia gravis (MG), reduced numbers or functionally impaired Treg cells have been reported. We have observed that PBL from myasthenic rats contain decreased numbers of CD4\textsuperscript{+}CD25\textsuperscript{high}Foxp3\textsuperscript{+} cells as compared with PBL from healthy controls, and we have tested whether Treg cells from healthy donors can suppress experimental autoimmune MG in rats. Because the number of naturally occurring Treg cells is low, we used an approach for a large-scale ex vivo generation of functional Treg cells from CD4\textsuperscript{+} splenocytes of healthy donor rats. Treg cells were generated ex vivo from CD4\textsuperscript{+} cells by stimulation with anti-CD3 and anti-CD28 Abs in the presence of TGF-β and IL-2. The obtained cells expressed high levels of CD25, CTLA-4, and Foxp3, and they were capable of suppressing in vitro proliferation of T cells from myasthenic rats in response to acetylcholine receptor, the major autoantigen in myasthenia. Administration of ex vivo-generated Treg cells to myasthenic rats inhibited the progression of experimental autoimmune MG and led to down-regulation of humoral acetylcholine receptor-specific responses, and to decreased IL-18 and IL-10 expression. The number of CD4\textsuperscript{+}CD25\textsuperscript{+} cells in the spleen of treated rats remained unchanged, but the subpopulation of CD4\textsuperscript{+}CD25\textsuperscript{high} cells expressing Foxp3 was significantly elevated. Our findings imply that Treg cells play a critical role in the control of myasthenia and could thus be considered as potential agents for the treatment of MG patients. The Journal of Immunology, 2008, 180: 2132–2139.

Myasthenia gravis (MG)\textsuperscript{1} is a disorder characterized by weakness and fatigability in which autoantibodies are generated against the acetylcholine receptor (AChR) at the neuromuscular junction, thereby impairing the transmission of signals from nerve to muscle. Experimental autoimmune MG (EAMG), induced in rats by immunization with Torpedo AChR, is a reliable model for the human disease and is suitable for investigating the mechanism(s) underlying the pathophysiology of myasthenia and for the development of novel therapeutic strategies. Although the symptoms of MG and EAMG are mediated primarily by autoantibodies, CD4\textsuperscript{+} T cells specific for the target autoantigen have a crucial role in the disease. Both Th1 and Th2 cells produce disease-associated cytokines, which are involved in the immunopathogenesis of MG and EAMG (1–3).

A subpopulation of suppressive CD4\textsuperscript{+} T cells, termed regulatory T (Treg) cells, was recognized to play a central and prominent role in the generation and maintenance of peripheral tolerance.

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\textsuperscript{2}Abbreviations used in this paper: MG, myasthenia gravis; AChR, acetylcholine receptor; EAMG, experimental autoimmune MG; Treg cell, regulatory T cell; evCD4\textsuperscript{+}CD25\textsuperscript{+}, ex vivo-generated CD4\textsuperscript{+}CD25\textsuperscript{+}; nCD4\textsuperscript{+}CD25\textsuperscript{+}, naturally occurring CD4\textsuperscript{+}CD25\textsuperscript{+}; nCD4\textsuperscript{+}CD25\textsuperscript{high}, naturally occurring CD4\textsuperscript{+}CD25\textsuperscript{high}.

Most endogenous CD4\textsuperscript{+} Treg cells constitutively express the CD25 molecule (IL-2 receptor α-chain). Natural CD4\textsuperscript{+}CD25\textsuperscript{+} Treg cells arise in the thymus and represent 5–10% of CD4\textsuperscript{+} T cells in the periphery. Treg cells mediate peripheral tolerance by suppressing the proliferation and cytokine production of effector autoreactive T cells that arise de novo or escape thymic deletion (4, 5). Treg cells specifically express Foxp3 (forkhead/winged helix transcription factor), which was reported to be essential for the development and functional activity of CD25\textsuperscript{+} Treg cells (6, 7). Subsequently, Foxp3 gene transfer was shown to convert naive CD4\textsuperscript{+}CD25\textsuperscript{−} T cells into a functional regulatory population, demonstrating the pivotal role of Foxp3 in Treg cell biology (8). The regulatory activity of CD4\textsuperscript{+}CD25\textsuperscript{+} T cells in peripheral blood was also associated with a high CD25 expression and CTLA-4 expression (9, 10).

The question of whether a dysfunction within the Treg compartment is involved in the pathogenesis of MG is of great interest. Several groups have evaluated peripheral blood cells and thymuses from patients to determine Treg number and function. These studies have found a normal to decreased population of CD4\textsuperscript{+}CD25\textsuperscript{high} cells in peripheral blood of MG patients compared with healthy controls (11–13). Following successful immunosuppression or thymectomy, CD4\textsuperscript{+}CD25\textsuperscript{high} cells of MG patients recover and reach normal or elevated numbers compared with healthy controls (11, 12). A functional impairment of thymic Treg cells was found in the thymus of MG patients and may thus be involved in the onset of the autoimmune process (14).

CD4\textsuperscript{+}CD25\textsuperscript{−} cells were found to be functionally involved in the suppressive action of various effective therapies demonstrated by us and by others in the mouse and rat experimental models for MG (15–20), suggesting that modulation of the CD4\textsuperscript{+}CD25\textsuperscript{−} cell compartment could play a key role in the treatment of myasthenia. The knowledge on Treg cell induction and activation opens the possibility of an attractive approach for immunotherapy of autoimmune diseases. However, with rare exceptions, Treg cell therapy
will require ex vivo expansion of cells, because regulatory T cells are present at low frequency in most sites that are clinically accessible for harvest.

In the present study, we analyzed PBL from myasthenic rats in an attempt to identify possible quantitative or qualitative defects in their regulatory T cell populations and found decreased numbers of CD4+ CD25+ Foxp3+ cells. We describe the ex vivo generation of Treg cells from CD4+ splenocytes of healthy donors and their in vitro functional characterization. Finally, we demonstrate that administration of such ex vivo-generated Treg cells from healthy donors to myasthenic rats suppresses EAMG possibly via an induction of de novo CD4+ CD25+ Foxp3+ regulatory T cells in the recipients.

Materials and Methods

Animals

Female Lewis rats 6–7 wk of age were obtained from the Animal Breeding Center of The Weizmann Institute of Science (Rehovot, Israel) and were maintained in the institute’s animal facilities. All the experiments in this study were performed according to the institutional guidelines for animal care.

Induction and clinical evaluation of EAMG

EAMG was induced in rats by injection of Torpedo AChR purified from the electric organ of Torpedo californica by affinity chromatography as described previously (21). Rats were immunized once in both hindfootpads by s.c. injection of Torpedo AChR (40 μg/rat) emulsified in CFA supplemented with additional nonviable Mycobacterium tuberculosis H37RA (0.5 mg/rat; BD Biosciences). Control rats were immunized with CFA supplemented with additional nonviable M. tuberculosis H37RA. Rats were monitored on alternate days for weight changes, and the clinical severity was graded 0–4, where grade 0 stands for healthy normal rats with no symptoms of EAMG; grade 1, mildly decreased activity, weak grip, with fatigability; grade 2, weakness, hunched posture at rest, decreased body weight, and tremor; grade 3, severe generalized weakness, significant decrease in body weight, moribund; and grade 4, dead (22). Clinical scores were recorded by double-blind evaluation for 7–8 wk following the immunization with Torpedo AChR. All experimental groups consisted of 8 rats unless otherwise specified, and all experiments were repeated two to three times.

Ex vivo generation of Treg cells and administration to rats

CD4+ T cells were purified from spleens of naive rats by negative selection, using rat CD4+ isolation kit (MagCellect, R&D Systems). Purity of obtained CD4+ cells was usually 88–92%. The purified CD4+ cells were cultured on 12-well Nunc plates precoated with 1 μg/ml anti-CD3 and 5 μg/ml anti-CD28 (Serotec) in a total volume of 1 ml of PBS and incubated overnight at 4°C or for 3 h at 37°C. Wells were then washed twice with 1 ml of PBS before plating the purified CD4+ cells at 4×10^5 cells/well in 2 ml of serum free X-VIVO-15 medium (BioWhittaker) supplemented with glutamine (2 mM), penicillin (20 U/ml), streptomycin (20 mg/ml), TGF-β (5 ng), and IL-2 (50 ng) (CytoLab). Twenty-four hours later, 10% FCS was added to the cultures. Cultures were incubated for 3–4 days at 37°C in 5% CO2. The resulting populations were analyzed using FACSscan (BD Biosciences).

For in vivo experiments, ex vivo-generated CD4+CD25+ cells (evCD4+CD25+), natural CD4+CD25+ cells (nCD4+CD25+), or PBS as control was administered i.p. into recipient rats starting 4 days after disease induction and every 2 weeks thereafter, up to a total of four injections. Three independent experiments were performed.

Functional in vitro assay of Treg cells

Cells differentiated as described above were tested for their ability to suppress proliferation of splenocytes. Splenocytes (5×10^5/well) removed from myasthenic rats 4–6 wk after disease induction were cocultured with evCD4+CD25+ cells (1×10^5/well) in X-VIVO-15 medium supplemented with glutamine (2 mM), penicillin (20 U/ml), streptomycin (20 mg/ml), and 0.5% normal rat serum. For control, splenocytes were cocultured with either fresh naturally occurring CD4+CD25+ cells (nCD4+CD25+) or nCD4+CD25+ cells (1×10^5/well) that were obtained from spleens of healthy rats and sorted by FACSsort. Torpedo AChR (0.125 μg/ml) was added to all wells. Proliferation in response to Torpedo AChR was assessed by measuring [3H]thymidine (0.5 μCi/well) incorporation during the last 18 h of a 5-day culture period. Results are expressed as mean cpm±SEM.

Anti-AChR Abs and Ab isotyping

Sera of treated rats were collected by retro-orbital bleeding 6 wk following disease induction. Anti-rat AChR Abs were determined by standard ELISA as follows: microtiter plates were coated with Rα1–205 (23), a recombinant fragment corresponding to the extracellular portion (amino acid residues 1–205) of the rat AChR α subunit (10 μg/100 μl in Tris-Cl (pH 8.0)), and reacted with 100 μl of rat sera at proper dilutions (1:1000 for total IgG, 1:50 for IgG1, 1:300 for IgG2a, 1:100 for IgG2b). The dilution points for each Ab were chosen within the linear range of the assay. Biotinylated mouse mAbs to rat IgG isotypes were added (1:100; Caltag Laboratories), followed by alkaline phosphatase-conjugated streptavidin, and Ab levels were evaluated by measuring the OD at 405 nm.

Real-time PCR

Primer sequences for cytokines, Foxp3, and CTLA-4 were previously described (20, 23). Real time RT-PCR (Roche LightCycler) was performed on cells derived from popliteal lymph nodes that were harvested from rats of all treated groups at the end of the experiment (7–8 wk following immunization with AChR) or on cultured/FACS-sorted CD4+ and CD4+CD25+ cells. Total RNA was isolated using the HiPure RNA isolation kit (Boehringer Mannheim) according to the manufacturer’s protocol and was reverse transcribed to prepare cDNA using Moloney murine

![FIGURE 1.](http://www.jimmunol.org/) Levels of CD4+ CD25+ cells in PBL of EAMG and healthy rats. Freshly isolated PBL from AChR- or CFA-immunized rats (3 wk following immunization) and from naive rats were analyzed by three-color flow cytometry for the expression of CD4, CD25, and Foxp3. The horizontal bars represent mean values. Shown are percentages of CD4+ cells expressing CD25+ (A), CD25+Foxp3- (B), and CD25+Foxp3+ (C), p < 0.05 for the differences between AChR-immunized rats and naive rats. (n = 5 for each group).

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leukemia virus reverse transcriptase (Promega). Obtained cDNA was subjected to real-time RT-PCR according to the manufacturer’s instructions with the following modifications: specific primer pairs were used to amplify specific genes in the presence of 3 mM of MgCl2. PCR was performed in duplicates in a total volume of 20 μl of LightCycler hot-start DNA SYBR Green I mix (Roche Diagnostics) containing primers and 5 μl of cDNA. PCR amplification was described before (20). The correlation coefficients for all curves were >1. Expression levels of -actin were used for normalization in calculating the expression levels of all other genes. The genes were also compared with another two housekeeping genes, L32 and GAPDH, and the results were found to be similar to those of -actin. Results are expressed as relative expression level for each gene.

Immuno-fluorescence flow cytometry

Flow cytometry analysis was performed on $2 \times 10^6$ purified CD4+ cells, evCD4+CD25+ cells or single-cell suspensions from naive, CFA, or AchR-immunized rats, and from spleens of treated myasthenic rats harvested at the end of the experiments. Cells were resuspended in FACS wash buffer (PBS, 0.1% BSA) and incubated with primary Abs to the tested cell-surface molecule (10 μg/ml) for 60 min at 4°C. Cells were washed and analyzed on a FACSscan flow cytometer. The following Abs were used for flow cytometry: PE-conjugated mouse IgG anti-rat CD4 (MCA55PE), PE-Cy5-conjugated mouse anti-rat CD4 (554839), and FITC-conjugated mouse IgG1 anti-rat CD25 (MCA273FT). Negative controls were: PE-conjugated mouse IgG1 (MCA1209PE), PE-Cy5-conjugated mouse IgG2a (35–4724-81), and FITC-conjugated mouse IgG1 (MCA1209F), all from Serotec, except for PE-Cy5-conjugated Abs, which were from eBioscience. Cells staining for Foxp3 were fixed and permeabilized before intracellular staining with PE-conjugated anti-Foxp3 (eBioscience) and were then stained for CD4 and CD25.

Statistics

Differences in mean values were compared between control and treatment groups by Student’s t test. A two-way ANOVA test was used to compare the control and the treatment groups all along the treatment. p values lower than 0.05 were considered to be significant.

Results

Number of circulating CD4+CD25+ cells in myasthenic rats is reduced

Several studies have shown a decreased population of CD4+CD25+ T cells or a functional impairment of these cells in MG patients as compared with healthy subjects (11–14). To identify possible regulatory T cell defects in EAMG, CD25 and Foxp3 expression was investigated on CD4+ T cells from PBL of myasthenic and control healthy rats. The percentage of CD4+CD25+ cells was lower in myasthenic (AChR-immunized) rats (mean, 6.07%; range, 4.23–7.58%) as compared with the two control groups comprised of naive rats (mean, 7.63%; range, 6.84–8.44%, p < 0.05) and CFA-immunized rats (mean, 6.91%; range, 5.72–9.26%) (Fig. 1A). Because regulatory activity of CD4+CD25+ T cells in peripheral blood was associated with high CD25 expression and Foxp3 expression (10), we focused on these subpopulations. We
found that the percentage of CD4<sup>+</sup>CD25<sup>high</sup> (Fig. 1B) and CD4<sup>+</sup>CD25<sup>+</sup>Foxp<sup>3</sup><sup>+</sup> (Fig. 1C) cells was lower in myasthenic rats (mean of 1.4 and 3%, respectively) as compared with healthy naive rats (1.77 and 4.01%, respectively, \( p < 0.05 \)). A lower percentage, although statistically not different, of CD4<sup>+</sup>CD25<sup>+</sup>Foxp<sup>3</sup><sup>+</sup> cells was also observed in myasthenic rats as compared with CPA-immunized rats.

Characterization of ex vivo-generated Treg cells

The observation that the number and/or activity of Treg cells in MG patients and in EAMG rats are different from their relevant healthy controls led us to test the possibility of treating myasthenic rats by functional Treg cells from healthy donors. However, the ability to use these cells therapeutically is limited by the small numbers of Treg cells resident in the circulation or in lymphoid organs of potential donors. It was therefore necessary to develop an approach for obtaining Treg cells in the large numbers required for their adoptive transfer into sick recipients. We used a rapid and efficient procedure to generate large numbers of CD4<sup>+</sup>CD25<sup>+</sup> cells from healthy control rats. CD4<sup>+</sup> splenic lymphocytes (88–92% purity) from naive rat donors, demonstrating a 5–8% prevalence of CD4<sup>+</sup>CD25<sup>+</sup> cells, were cultured for 3 days on anti-CD3 and anti-CD28 Ab-coated tissue culture plates in the presence of IL-2 (50 ng) and TGF-β (5 ng). The phenotype of the cells was examined on day 0 and after 3 days in culture by flow cytometry and real-time RT-PCR. FACS analysis of the cells after 3 days in culture showed a content of 94–96% CD4<sup>+</sup>CD25<sup>+</sup> cells as compared with a prevalence of 5% of these cells on day 0 (Fig. 2A). Quantitative RT-PCR analysis revealed a significant up-regulation in the mRNA levels of Foxp3 (33-fold increase) and CTLA-4 (4.75-fold increase) in the evCD4<sup>+</sup>CD25<sup>+</sup> as compared with the nCD4<sup>+</sup>CD25<sup>+</sup> cells. TGF-β mRNA levels did not increase in the evCD4<sup>+</sup>CD25<sup>+</sup> cells and were similar to their levels in CD4<sup>+</sup>CD25<sup>+</sup> cells (Fig. 2B).

To determine whether ex vivo-generated Treg cells exhibit normal suppressive functions, we evaluated their ability to modulate the proliferation of splenocytes from myasthenic rats in response to AChR (0.125 μg/ml). Splenocytes isolated from EAMG rats were mixed at a ratio of 5:1 with evCD4<sup>+</sup>CD25<sup>+</sup> cells and with either nCD4<sup>+</sup>CD25<sup>+</sup> or nCD4<sup>+</sup>CD25<sup>−</sup> cells freshly isolated from naive rats. The evCD4<sup>+</sup>CD25<sup>+</sup> cells and the freshly isolated nCD4<sup>+</sup>CD25<sup>−</sup> Treg cells produced a suppression of 37 ± 11% and 50 ± 17%, respectively, whereas the nCD4<sup>+</sup>CD25<sup>−</sup> cells freshly isolated from naive rats did not exert any suppression (Fig. 2C). Suppression by the evCD4<sup>+</sup>CD25<sup>+</sup> cells and by the nCD4<sup>+</sup>CD25<sup>−</sup> cells freshly isolated from naive rats was not statistically different from each other.

Ex vivo-generated Treg cells from healthy rats suppress EAMG

To test the potential of the evCD4<sup>+</sup>CD25<sup>+</sup> cells to affect the course of EAMG, these cells were administered i.p. into recipient rats (3–6 × 10<sup>6</sup>/rat) 4 days after disease induction. Three additional similar administrations were given at 2-wk intervals. Control rats received either nCD4<sup>+</sup>CD25<sup>−</sup> cells isolated from naive rats or PBS administered in the same manner. The ex vivo-generated Treg cells had a suppressive effect on EAMG (Fig. 3). Eight weeks after disease induction, the mean clinical score (0.66; \( n = 30 \)) of rats treated with evCD4<sup>+</sup>CD25<sup>+</sup> cells was significantly lower than those of the control nCD4<sup>+</sup>CD25<sup>−</sup>-treated rats or PBS-treated rats (1.22 and 1.65, respectively; \( n = 16 \) for each group). Fig. 3A presents the mean clinical scores in a representative experiment. In the group of rats treated by evCD4<sup>+</sup>CD25<sup>+</sup> cells, 16 of 30 (53%) did not show any clinical symptoms of EAMG 8 wk after disease induction (Fig. 3B). At the same time point, only 1 of 16 (6.25%) of PBS-treated rats and 4 of 16 (25%) of nCD4<sup>+</sup>CD25<sup>−</sup>-treated control rats did not show any symptoms of EAMG (Fig. 3B). Rats treated by the evCD4<sup>+</sup>CD25<sup>+</sup> cells gained weight during the experiment, whereas rats treated similarly by either nCD4<sup>+</sup>CD25<sup>−</sup> or PBS lost weight from 4 wk on (data not shown).

Although the number and/or function of Treg cells of MG patients (11–14) and of EAMG rats (Fig. 1) are impaired as compared with Treg cells in healthy controls, in clinical terms, administration of autologous cells may have an apparent advantage. We have therefore attempted to treat EAMG rats also by CD4<sup>+</sup>CD25<sup>+</sup> cells generated from AChR-immunized rats. Treg cells from CD4<sup>+</sup> splenocytes derived from AChR-immunized donors, harvested 4–6 wk after disease induction, were generated by the same protocol as described above for cells from healthy donors. The phenotype of the generated cells was examined after 3 days in culture by FACS analysis, and the purity of CD4<sup>+</sup>CD25<sup>+</sup> cells was >90%. However, the expression of CD25<sup>high</sup> was 6.2-fold lower in the cells generated from CD4<sup>+</sup> cells of myasthenic rats as compared with those generated from CD4<sup>+</sup> cells of healthy donors. Treg cells were administered to EAMG rats in a similar protocol to the one described above. We found that in contrast to evCD4<sup>+</sup>CD25<sup>+</sup> cells from healthy donors, evCD4<sup>+</sup>CD25<sup>+</sup> cells from myasthenic donors were not effective in suppressing EAMG and even resulted in exacerbation of disease. The mean clinical score of rats treated with evCD4<sup>+</sup>CD25<sup>+</sup> cells (1 × 10<sup>6</sup>/rat) derived from AChR-immunized rats was 3.4 when monitored 11 wk after disease induction, as compared with 2.4 in PBS-treated rats. Under these conditions, rats treated by evCD4<sup>+</sup>CD25<sup>+</sup> cells from healthy donors had a clinical score of 2.1.

Rats treated by ex vivo-generated Treg cells have a reduced humoral AChR-specific response

The anti-AChR Ab titers in sera withdrawn from treated rats 6 wk following disease induction were determined by ELISA with a rat
recombinant fragment and with the immunizing Torpedo AChR. The anti-rat AChR Ab titer and the specific anti-AChR IgG iso-
types were measured in sera withdrawn from treated rats 6 wk
following disease induction. As shown in Fig. 4, the levels of
anti-rat AChR IgG and of IgG1, IgG2a, and IgG2b were signifi-
cantly lower in rats treated by evCD4\textsuperscript{+}CD25\textsuperscript{+}
cells as compared with control PBS-treated rats. This pattern of reduction in both
IgG1 and IgG2 anti-AChR IgG isotypes suggests that treatment
with Treg cells suppresses Th1- as well as Th2-regulated anti-
AChR responses. Note that no significant differences, except for
lower levels of AChR-specific IgG2b, were detected in rats treated
by nCD4\textsuperscript{+}CD25\textsuperscript{+} cells.

The anti-Torpedo Ab titer in rats treated by evCD4\textsuperscript{+}CD25\textsuperscript{+} was also reduced as compared with the titer in control rats, but the
reduction was less marked. We assume that only a minority of the
Abs elicited following immunization with Torpedo AChR cross-
react with rat muscle AChR and are hence pathogenic.

Administration of Treg cells leads to reduced Th1- and Th2-type
cytokine expression

Treg cells were previously shown to act by suppressing normal
CD4\textsuperscript{+} T cell activation and Th1 and Th2 cytokine production,
thereby playing an important role in the control of autoimmune
diseases (24–26). Real-time RT-PCR was used to determine
mRNA levels of IL-18, IL-10, and TGF-\beta in lymph node cells of
rats treated with evCD4\textsuperscript{+}CD25\textsuperscript{+} and with either PBS or
nCD4\textsuperscript{+}CD25\textsuperscript{+} as controls. As shown in Fig. 5, treatment by ex
vivo-generated Treg cells resulted in down-regulation of the Th1-
type cytokine IL-18 and of the Th2-type cytokine IL-10. The

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** Effect of treatment by ex vivo-generated Treg cells on the
humoral anti-AChR response. Sera were obtained from rats 6 wk after
disease induction and were analyzed by ELISA for total anti-AChR titer
and for isotypes of anti-AChR IgG Abs. Error bars indicate SEM values
\((n = 8)\). *, \(p < 0.05\).

![Figure 5](http://www.jimmunol.org/)

**FIGURE 5.** Effect of treatment by ex vivo-generated Treg cells admin-
istration on cytokines. Lymph node cells were obtained from rats 8 wk after
disease induction (~10 days after last cell administration) and mRNA ex-
pression levels of cytokines were analyzed by quantitative real-time RT-
PCR. \(\beta\)-actin was used as an inner control for normalization for each cy-
tokine. Error bars indicate SEM values \((n = 8)\). *, \(p < 0.05\).
To find out whether administration of evCD4 was significantly increased in rats treated by evCD4 B6 (mean 2.7%) as compared with PBS-treated rats (mean 1.7%) (Fig. 6C), mRNA expression levels of the Th3-type cytokine, TGF-β, did not change significantly.

**Discussion**

The identification of CD4⁺CD25⁺Foxp3⁺ Treg cells as important regulators of tolerance has opened a major area of investigation raising the possibility that a dysfunction in the Treg compartment is involved in the etiology and pathogenesis of autoimmune diseases. In this study we demonstrated a reduction in the number of these cells in the rat experimental model of MG, in line with prior studies demonstrating abnormalities in the number and/or function of these cells in myasthenia. These observations in MG and EAMG suggest that augmentation of the regulatory T cell population could lead to improved control of the autoimmune process. Our data show that adoptive transfer of ev vivo-generated Treg cells has a suppressive effect on the progression of EAMG and suggest that a therapeutic modality based on this approach could be developed for the treatment of MG.

Peripheral blood cells and thymuses from patients have been previously shown to contain a decreased population of CD4⁺CD25⁺ T cells compared with healthy controls (11–13). The CD4⁺CD25⁺ T cells recover and sometimes even become more numerous than in healthy controls when patients are successfully immunosuppressed or undergo thymectomy (11, 12). A functional impairment of Treg cells was also found in the thymus of MG patients and may thus be involved in the onset of the autoimmune process (14). These changes in thymic or peripheral Treg cells may result from genetic or environmental insults, which can alter the delicate balance between Treg cells and self-reactive T cells, thus provoking a developmental or functional deficiency in Treg cells, and ultimately leading to autoimmunity. As most Treg cells cycle continuously in the periphery in response to self-Ag recognition, it is possible that they are inherently more sensitive than other T cells to certain environmental triggers. In various rodent systems, it has been shown that physical, chemical, and biological agents or genetic aberrations can indeed cause autoimmune disease by affecting the size of the peripheral Treg cell pool as well as thymic T-cell output and thymic selection (27). The reduced numbers of circulating Treg cells observed in our study may be due to proinflammatory cytokines, which are known to be elevated in EAMG. Wu et al. reported that TNF-α reduces the number of CD4⁺CD25⁺ T cells in the thymus and spleen of NOD mice (28). Bettelli et al. recently reported that IL-6 could completely inhibit the TGF-β-induced generation of Foxp3⁺ Treg cells. In combination with IL-6, TGF-β was shown to promote the production of the pathogenic Th17 cells rather than elicitation of Foxp3⁺ Treg cells (29).

We show that administration of Treg cells generated from healthy donors is capable of suppressing EAMG. Interestingly, administration of CD4⁺CD25⁺ T cells generated from myasthenic rats did not suppress EAMG in the recipients, and in some cases such administration even led to exacerbation of disease. This observation suggests that the CD4⁺CD25⁺ population in EAMG is also qualitatively different from its counterpart in healthy rats and may reflect the presence of elevated numbers of activated effector cells and lower numbers of regulatory cells in the CD4⁺CD25⁺ population of myasthenic rats.

Treatment by exogenous Treg cells depends on the availability of sufficient numbers of these cells. However, naturally occurring Treg cells are present at low frequencies in most sites that are clinically accessible for harvest. This implies that Treg cell-based
therapy will require ex vivo expansion of cells. Two main approaches have been proposed for expanding the unselected regulatory T cell population. In the first one, studies have shown that Treg cells can be expanded in vitro by “pushing” CD4+CD25+ cells from humans and mice into division by CD3- and CD28-specific Abs in combination with high doses of IL-2. Under these conditions, Treg cells can be expanded within 2–3 wk (30–32). The other approach used CD4+CD25+ cells as the source for Treg cells, cultivating them in the presence of Treg cell-promoting cytokines, or transfecting them with genes that promote Treg cell development (33–36). In this study we used a rapid and efficient method to generate large numbers of CD4+CD25+ cells within 3 days by combining these two approaches. Because it has been shown that natural and induced CD4+CD25+ cells can educate CD4+CD25− cells in vitro to turn into CD4+CD25+ Treg cells and to develop suppressive activity (37), we generated CD4+CD25+ starting from a full population of CD4+ cells. Splenic CD4+ cells were stimulated with anti-CD3 and anti-CD28 Abs in the presence of IL-2 and TGF-β. This was based on studies demonstrating that IL-2 and TGF-β together induce CD4+ cells to become suppressor cells and enhance their viability in vitro and in vivo (34, 38, 39). Besides inducing naive cells to turn into Treg cells, the combination of IL-2 and TGF-β enhances both the in vitro and in vivo suppressive effects of mature CD4+CD25+ Treg cells (40). Taylor et al. reported that allostimulation of mouse Treg cells with IL-2 and TGF-β and transfer of only 5 × 10^5 of these cells to mice protected them from acute graft-vs-host disease (41). Our ex vivo-generated Treg cells exerted functional characteristics of freshly isolated CD4+CD25+ cells and expressed Foxp3, CTLA-4, and CD25high, all of which were implicated as important for Treg function.

The ultimate therapeutic potential of Treg cells depends on the ability to treat individuals with ongoing disease. In this study, we demonstrated that ex vivo-generated Treg cells suppress the clinical symptoms of ongoing EAMG and inhibit the progression of the disease. Treg cells possess several immunological features relevant to their key role in suppression of autoimmunity. It is well documented that CD4+CD25+ T cells inhibit the proliferation and cytokine production by both Th1 and Th2 cells in vitro and in vivo (42, 43) and possibly also suppress B cells (9). Indeed, administration of the ex vivo-generated Treg cells resulted in a reduction of Th1 and Th2 cytokine expression, reflected in suppressed mRNA levels of IL-18 and IL-10 and reduction in the humoral AChR-specific response reflected in decreased IgG1 levels, which in the rat is regulated by Th2-type cells and IgG2a and IgG2b levels that are regulated by Th1 cells.

Among the most interesting and perhaps unexpected observations in this study was the content of Treg cells among splenocytes of treated rats. The percentage of CD4+CD25+ cells was essentially the same in rats treated by Treg cells or PBS. However, the number of CD4+CD25+ cells expressing Foxp3 was significantly increased in rats treated by Treg cells as compared with PBS-treated rats. Although we cannot rule out the possibility, it is unlikely that the elevated CD4+CD25+ Foxp3+ cells observed in the treated rats were of donor origin because only a specific subpopulation of CD4+CD25+ cells was elevated and because it was shown that only 20% of administered Treg cells are located in the spleen (44). Induction of de novo Treg cells in the recipients was recently reported by Zheng et al. (45), who demonstrated transfer of regulatory T cells to modify graft rejection through induction of tolerogenic CD4+CD25+ cells that were of recipient origin. These observations provide evidence that ex vivo-generated Treg cells can educate recipient CD4+ cells to become regulatory cells that express Foxp3 and have suppressive activity.

The role of Ag specificity in the function of Treg cells in tolerance control is still controversial (46). Our observations suggest that adoptively transferred polyclonal Treg cells from healthy donors suppress EAMG and may act via generating new, possibly Ag-specific, CD4+CD25+Foxp3+ regulatory T cells in the recipients. Although polyclonal Treg cells were effective in suppressing ongoing EAMG, additional experiments should be performed to address the question of whether Ag-specific Treg cells have an advantage over the polyclonal Treg cells used in this study.

In summary, our data show the involvement of CD4+CD25+ Treg cells in EAMG and demonstrate the therapeutic potential in EAMG of ex vivo-generated polyclonal Treg cells generated from CD4+ cells of healthy donors. This therapeutic potential of CD4+CD25+ cells may thus represent an attractive approach for immunotherapy of MG.

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Disclosures

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References