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Naturally Occurring CD4+CD25+ Regulatory T Cells Prevent but Do Not Improve Experimental Myasthenia Gravis

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In the current study, we investigated whether naturally occurring CD4+CD25+ T cells, separated by immunomagnetic anti-CD4 and anti-CD25 Abs from naive animals, are able to protect from experimental autoimmune myasthenia gravis (EAMG) and modify the progression of ongoing disease when administered to *Torpedo californica* acetylcholine receptor (AChR)-immunized Lewis rats. Even though CD4+CD25+ and CD4+CD25high T cell frequencies were similar in the spleens and lymph nodes of EAMG and healthy rats, we observed that CD4+CD25+ T cells isolated from the spleens of naive animals inhibited in vitro the Ag-induced proliferation of T cell lines specific to the self-peptide 97–116 of the anti-AChR subunit (R97-116), an immunodominant and myasthenogenic T cell epitope, whereas CD4+CD25+ T cells purified from the spleens of EAMG rats were less effective. CD4+CD25+ T cells from EAMG rats expressed less forkhead box transcription factor P3 but more CTLA-4 mRNA than healthy rats. Naive CD4+CD25+ T cells, obtained from naive rats and administered to *T. californica* AChR-immunized animals according to a preventive schedule of treatment, reduced the severity of EAMG, whereas their administration 4 wk postinduction of the disease, corresponding to the onset of clinical symptoms (therapeutic treatment), was not effective. We think that the exogenous administration of CD4+CD25+ naive T cells prevents the early events underlying the induction of EAMG, events linked to the T cell compartment (Ag recognition, epitope spreading, and T cell expansion), but fails to ameliorate ongoing EAMG, when the IgG-mediated complement attack to the AChR at the neuromuscular junction has already taken place. *The Journal of Immunology*, 2010, 185: 5656–5667.

Myasthenia gravis (MG) is a B cell-mediated, T cell-dependent autoimmune disorder in which the major autoantigen is the nicotinic acetylcholine receptor (AChR) at the neuromuscular junction. Patients with MG show muscle weakness and fatigability, the clinical expression of impaired neuromuscular transmission caused by IgG autoantibodies binding to the AChR on the postsynaptic membrane of skeletal muscle (1, 2). Experimental autoimmune MG (EAMG) mimics human MG in its clinical and immunological manifestations and is considered a reliable model to investigate therapeutic strategies for treatment of the human disease (3, 4). EAMG can be induced in susceptible rat strains by immunization with AChR purified from *Torpedo californica* electroplax AChR (TAChR) in CFA (5). Cellular and molecular mechanisms that underlie the breakdown of self-tolerance and the development of self-reactive anti-AChR Abs in both EAMG and its human counterpart remain partially

unknown. Evidence accumulated in recent years suggested the existence of a subset of CD4+ T cells, named naturally occurring regulatory T cells (Tregs), that exerts immune suppressive activity and plays a significant role in the maintenance of peripheral immunological tolerance (6). Naturally occurring Tregs constitutively express the CD25+ molecule (IL-2R α-chain), arise in the thymus, and represent 5–10% of CD4+ T cells in the periphery (7–10). This T cell subpopulation plays an essential role in maintenance of immunologic self-tolerance and a negative control on a variety of physiological and pathological immune responses. Tregs are anergic in vitro and suppress proliferation and cytokine production of CD4+ effector T cells (Teffs) in response to different polyclonal stimuli (11). Furthermore, Tregs specifically express forkhead box transcription factor P3 (FoxP3) that has a major role not only in the development but also in the characterization of Treg biology (12). Deleterious alteration of Tregs results in the onset of autoimmune diseases in a variety of animal models, resembling the corresponding human disorders. Many studies have shown that FoxP3 expression tends to decrease in human autoimmune diseases, such as juvenile idiopathic arthritis (13) and psoriasis (14). A reduction in Treg number and a functional defect in their in vitro suppressive activity have been described in MG (15–18); moreover, their role and potential therapeutic use in EAMG have been investigated (10, 19, 20). Aricha et al. (10) reported the effect of in vitro-generated Treg-like cells (stimulated by anti-CD3 and anti-CD28 Abs in the presence of TGF-β) on prevention of EAMG. They demonstrated that in vitro-induced Treg-like cells (from healthy rats) suppressed EAMG when administered immediately after TAChR immunization, lasting for the whole duration of the study. Given the pivotal role of naturally occurring naive Tregs in maintaining a condition of self-tolerance, we investigated whether the CD4+CD25+ T cell subset is functionally altered in EAMG rats and whether the administration of

CD4$^+$CD25$^+$ T cells from healthy animals can prevent EAMG or improve the clinical symptoms of the already established disease.

Materials and Methods

Animals

Female Lewis rats, 6–8 wk of age, were purchased from Charles River Laboratories (Calco, Italy) and housed at the animal facility of the Neurological Institute Foundation Carlo Besta (Milan, Italy). Experiments were approved by the Ethic Committee of the Institute and performed in accordance with the Principles of Laboratory Animal Care (European Communities Council Directive 86/609/EEC). Animals were sacrificed after deep anesthesia obtained by carbon dioxide; low-grade anesthesia with 4% chloral hydrate (Sigma-Aldrich, St. Louis, MO) administered i.p. was used for immunization and treatments.

Antigens

AChR was purified from *Torpedo californica* electroplax tissue (Aquatic Research Consultants, San Pedro, CA) by affinity chromatography on *Naja*-naja siamensis toxin coupled to Sepharose 4B (GE Healthcare Life Sciences, Piscataway, NJ) (21). The TAChR concentration was determined by [^{125}I]antibungarotoxin (anti-BTX; PerkinElmer, Waltham, MA) binding assay, and the sp. act. was 3.7–5.5 pmol [^{125}I]anti-BTX binding sites/mg protein. The purified receptor was analyzed on SDS-PAGE. TAChR preparations were dialyzed extensively against 0.01 M Tris-HCl (pH 7.5), 0.001 M EDTA, 0.1 M NaCl (Tris-HCl buffer), and 0.1% Triton X-100 (all from Sigma-Aldrich). TAChR used for T cell proliferations was further dialyzed against Tris-HCl buffer plus 0.025% Triton X-100 and filter-sterilized (0.2 μm). Peptide R97–116, corresponding to region 97–116 of the rat AChR α-subunit, and peptide T97–116 (from torpedo AChR) were synthesized by Dr. R. Longhi (C.N.R., Milan, Italy), according to sequences already published in GenBank (http://www.ncbi.nlm.nih.gov/genbank/; accession number X74832 for rat AChR, sequence DGDFAIVKFTKVLLDYTGHI; accession number J00963 for *Torpedo* AChR, sequence DGDFAIVHMTKLLLDYTGKI). Peptides were synthesized using F-moc chemistry on a 431A automated peptide synthesizer (PE Applied Biosystems, Foster City, CA). Peptides were purified by reverse-phase HPLC; the synthesis was confirmed by mass spectroscopy. Chicken egg white albumin (OVA; Sigma-Aldrich) was used to prime Lewis rats by immunization in the hind footpads (100 μg in CFA supplemented with 1 mg/rat H37Ra; both from Difco Laboratories, Detroit, MI).

EAMG induction and treatment protocols

EAMG was induced in female Lewis rats (6–8 wk) by immunization in the hind footpads with 50 μg purified TAChR in CFA (Difco Laboratories) supplemented with 1 mg/rat H37Ra (Difco Laboratories). Peptide R97–116 was used instead of TAChR to induce Ag-specific T cell lines needed for in vitro or ex vivo experiments.

The efficacy of CD4$^+$CD25$^+$ treatments was initially evaluated on TAChR- and R97–116-primed Lewis rats. TAChR- or R97–116-immunized rats received a single dose of CD4$^+$CD25$^+$ cells 24 h postimmunization (1, 2, and 4 × 10^6 CD4$^+$CD25$^+$ cells/animal, i.p.) and sacrificed 10 d postimmunization. Single-cell suspensions from draining lymph nodes (LNs) were then challenged in vitro with mitogen and TAChR or R97–116 (see below). Two protocols were adopted to assess the efficacy of CD4$^+$CD25$^+$ treatments on EAMG, as follows: 1) preventive protocol, starting at day 7 postimmunization, with 2–2.5 × 10^6 CD4$^+$CD25$^+$ T cells in sterile saline solution administered i.p., once a week, four times; and 2) therapeutic protocol, starting at week 4 postimmunization (corresponding to the onset of clinical signs of EAMG) with 2–2.5 × 10^6 CD4$^+$CD25$^+$ T cells administered four times and with 4 × 10^6 CD4$^+$CD25$^+$ T cells administered five times. The control groups received vehicle only. TAChR-immunized rats were randomly assigned to the different treatment arms by an operator blinded to EAMG scores.

EAMG clinical evaluation

Evaluation of disease manifestations in TAChR-immunized rats was performed by testing muscular weakness. Clinical scoring was based on the presence of tremor, hunched posture, low muscle strength, and fatigability and assessed after exercise (repetitive paw grips on the cage grid) for 30 s. Disease severity was graded as follows: grade 0, normal strength and no fatigability; grade 1, mildly decreased activity and weak grip or cry; grade 2, clinical signs present at rest; grade 3, severe clinical signs at rest, no grip, moribund; and grade 4, dead (22). Each animal was weighed and evaluated twice weekly for the entire duration of the experiments; EAMG was confirmed by edrophonium chloride (Sigma-Aldrich) test. Results are expressed as the mean of the evaluations for each animal at each time point.

Purification of CD4$^+$CD25$^+$ T cells

Spleens were aseptically removed from healthy, EAMG (TAChR/CFA-immunized), and OVA/CFA-immunized rats and processed into a single-cell suspension. RBCs were lysed with cold distilled water. CD4$^+$ cells were purified by incubation with FITC-conjugated anti-CD4 mAb (clone OX-35, BD Biosciences, San Jose, CA) for 10 min at 4°C, followed by incubation with anti-FITC magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for a further 15 min. CD4$^+$ cells were separated on an LS column (Miltenyi Biotec) according to the manufacturer's instructions. Postelution of CD4$^+$ cells, magnetic microbeads were released by incubation in specific buffer according to the manufacturer's instructions. CD4$^+$ enriched cells were then stained with PE-conjugated anti-CD25 mAb (clone OX 39, BD Pharmingen) for 10 min at 4°C, followed by incubation with anti-PE magnetic microbeads (Miltenyi Biotec) for a further 15 min. CD4$^+$CD25$^+$ cells were separated by selection on an MS column (Miltenyi Biotec). Magnetic microbeads were released by incubation in specific buffer according to the manufacturer's instructions. Cells were left in tissue culture medium (see below) for at least 2 h, at 37°C 5% CO$_2$, before any further use.

Immunophenotyping by flow cytometry

Different combinations of Abs were used to characterize cells derived from EAMG and healthy rats. The following Abs, directly conjugated to FITC or PE, were used: CD3 (clone G4.18), CD4 (clone OX-35), CD8 (clone 341), CD25 (clone OX 39, all from BD Biosciences), FoxP3 (clone 150D, Biolegend, San Diego, CA), and CTLA-4 (clone WKH203, Biolegend). For flow cytometric analysis, typically 3 × 10^5 cells were stained with 10 μl appropriated diluted Ab for 30 min at 4°C, washed twice in cold PBS 1% FCS, and analyzed on the FACSCalibur (BD Biosciences). For the analysis of intracellular FoxP3 and CTLA-4 staining, cells were initially incubated with the appropriated Ab for the membrane Ag, fixed, and permeabilized using IntraPrep Permeabilization reagents (Immunotech, Praha, Czech Republic), and then labeled with biotinylated anti-FoxP3 or anti–CTLA-4 Ab.

Real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized from RNA using random hexamers (GE Healthcare Life Sciences) and reverse transcriptase (Moloney murine leukemia virus; Life Technologies, Carlsbad, CA). Real-time quantitative PCR (qPCR) for IFN-γ, IL-10, IL-6, FoxP3, and CTLA-4 was performed using Assay-on Demand Gene Expression Products (Applied Biosystems, Foster City, CA). β-Actin and GAPDH (Applied Biosystems) were used as endogenous control, without any substantial differences in the relative expression of target genes.

Real-time PCR reactions of each gene were performed in triplicate with an ABI Prism 7500 FAST Real-Time PCR System (Applied Biosystems). Levels of mRNA expression for each gene were calculated using the $2^{-\Delta Ct}$ method, in which ΔCt represents the difference between the cycle threshold (Ct) of the target gene and Ct of the housekeeping gene. Data are represented as $2^{-\Delta Ct} \times 100$ (23).

T cell lines culture and proliferative responses

T cell lines were grown from lymph nodes of R97–116 immunized rats by Ag-specific stimulation with 5 μg/ml R97–116 peptide in RPMI 1640 medium plus 10% FCS, 1% sodium pyruvate, 1% nonessential amino acids, 1% L-glutamine, 1% penicillin-streptomycin (Euroclone Celbio, Milan, Italy), and 2 × 10^{-5} M 2-ME (BDH, West Chester, PA) (tissue culture medium). T cell lines were then expanded with rIL-2 (10 U/ml; Roche, Basel, Switzerland) every 3 to 4 d and restimulated with the appropriate Ag every 2 wk. For proliferation assay, 3 × 10^4 T cells or CD4$^+$CD25$^+$ cells were cultured in triplicate with irradiated (3000 cGy) splenic cells as APCs/feeder cells (2 × 10^5/well) plus the relevant Ag. ConA (Sigma-Aldrich) was used at 2 μg/ml as positive control. CD4$^+$CD25$^+$ T cells from EAMG or healthy rats were cocultured with 3 × 10^4 R97–116 T cell lines at different CD4$^+$CD25$^+$ cell/R97–116 T cell ratios: 1:1, 0.2:1, and 0.1:1, in the presence of APCs (2 × 10^5 cells). After 72 h, 0.5 μCi [^{3}H]thymidine (GE Healthcare Life Sciences) was added to each well; cells were harvested after a further 16–18 h incubation. [^{3}H]thymidine incorporation was evaluated on a Wallac MicroBeta TriLux counter (PerkinElmer). Results are expressed as mean cpm ± SE of triplicate cultures.

LN proliferative responses

LNs were removed from healthy and immunized Lewis rats and processed into a single-cell suspension; LN cells (LNCs; 2×10^5/well) were cultured with TAChR (1.25 and 0.25 µg/ml), R97–116 or T97–116 peptides (5–10 µg/ml), ConA (2 µg/ml) in RPMI 1640 medium plus 1% normal rat serum. Cells were tested alone and in cocultures at a 0.2:1 CD4⁺CD25⁺ cells/LNCs ratio (4×10^4:20×10^4). After 72 h of culture, 0.5 µCi [³H] thymidine (GE Healthcare Life Sciences) was added to each well, and [³H] thymidine incorporation was evaluated after an additional 16–18 h. Results are expressed as mean cpm ± SE.

Multiparametric cytokine assay

INF-γ, IL-10, and IL-6 production from TAChR-stimulated (1.25 µg/ml) LNCs was evaluated by commercially available multiparametric cytokine assay (R&D Systems, Minneapolis, MN) and analyzed on the Bio-Plex 200 System (Bio-Rad, Hercules, CA). LNCs (2×10^6/well) were incubated for 72 h and the supernatants harvested and stored at −20°C, pending the assay. Microparticles precoated with anti–rINF-γ Ab (LUR585), anti–rIL-10 Ab (LUR522), and anti–rIL-6 Ab (LUR506, all from R&D Systems) were used and incubated with culture supernatants accordingly to the manufacturer's instructions. Calibration curves were done with standard reagents provided in the assay. Cytokine concentration is expressed as picograms per milliliter.

Assay of anti-rat AChR Abs

Anti-rat AChR Abs were assayed in individual sera by conventional radioimmunoprecipitation method (22, 24). Briefly, rat AChR was extracted from healthy rat muscle and labeled with 2×10^{-9} M [¹²⁵I]anti-BTX. Experimental and control sera were incubated overnight (ON) with [¹²⁵I]anti-BTX rat AChR (0.5 pmol); Ab–AChR complexes were precipitated by adding an excess of rabbit anti-rat IgG (Sigma-Aldrich). The precipitate was washed twice with 1 ml cold PBS 0.5% Triton X-100 and [¹²⁵I]anti-BTX labeling evaluated in a gamma-counter. A parallel assay was carried out in the presence of rat AChR preincubated in excess of unlabeled (cold) anti-BTX to eliminate aspecific binding rate. The anti-AChR Ab titers were expressed as picomoles of [¹²⁵I]anti-BTX binding sites precipitated per milliliter of serum.

ELISA of anti-T. californica AChR Ab and isotypes

Anti-TAChR IgG was determined qualitatively using a standard ELISA (25). Briefly, 96-well microtiter plates were coated ON with 1 µg purified TAChR at 4°C, then quenched with PBS, 0.05% Tween 20, and 1% BSA at 37°C for 1.5 h. Rat sera, diluted 200-fold in PBS/Tween 20, 1% BSA, were incubated for 2 h at room temperature. Plates were washed and diluted (1:10,000), and rabbit anti-rat IgG (HRP-conjugated; Sigma-Aldrich) was added. IgG subclasses were revealed using mouse anti-rat mAbs (1:250) specific for each subtype (IgG1, IgG2a, and IgG2b from Sigma-Aldrich and IgG2c from BD Pharmingen) after 1 h incubation at room temperature followed by incubation for 1 h with diluted (1:10,000) anti-mouse IgG (HRP-conjugated; Sigma-Aldrich), and revealed by tetramethylbenzidine substrate. OD was measured at 450 nm using an automated microplate ELISA reader. Each serum was tested in duplicate. Results are expressed as OD at 450 nm.

AChR content in rat muscle

AChR content in muscles was assayed as previously described (26). AChR was solubilized from muscle membranes with Tris-HCl buffer, 2% Triton X-100, ON, at 4°C, and the solutions containing AChR clarified by centrifugation at $100,000 \times g$ for 30 min. AChR crude extracts (100 µl, duplicates) were incubated with [¹²⁵I]anti-BTX 4 h at room temperature, transferred on DE-81 DEAE disks (Whatman International, Kent, U.K.), and washed with Tris-HCl buffer 0.5% Triton X-100. Radioactivity was determined by γ-counting. The aspecific binding was calculated on parallel samples preincubated with unlabeled anti-BTX. The results were expressed as femtomoles of toxin-binding sites per gram of muscle.

Statistical analysis

ANOVA, with Dunnett's multiple comparison post hoc test, or two-tailed t test was performed to assess statistical significance of the results. Differences were considered significant when $p < 0.05$. Statview 5 for Macintosh (Abacus Concepts, Berkeley, CA) and GraphPad Prism version 4.0 for Macintosh (GraphPad, San Diego, CA) were used for data elaboration.

Results

LN and spleen CD4⁺CD25⁺ frequencies are similar in EAMG and normal healthy rats

A recent study on the EAMG rat model reported a functional impairment of CD4⁺CD25⁺ T cells in the peripheral blood of EAMG animals compared with controls (10). To identify if such differences were also present in secondary lymphoid organs, we studied CD4⁺ (Th), CD4⁺CD25⁺ (Th regulatory), CD8⁺ (T cytotoxic), and CD8⁺CD25⁺ (T cytotoxic regulatory) (27) cell subsets in draining LNs and spleens from EAMG ($n = 6$; mean clinical score, 2.5 ± 0.6) and healthy ($n = 5$) rats by FACS analysis; data are reported in Table I, and a representative FACS analysis of cells isolated from an EAMG rat and a healthy animal are reported in Fig. 1. Cell suspensions from spleens gave similar yields (mean cell number ± SE, $188.4 \pm 15.2 \times 10^6$ EAMG; $193.3 \pm 23.5 \times 10^6$ healthy animals), whereas cell recovery from draining (popliteal and inguinal) LNs was different in EAMG (mean cell number ± SE, $80.3 \pm 11.1 \times 10^6$) compared with healthy rats (mean cell number ± SE, $5.0 \pm 0.8 \times 10^6$), as a consequence of TAChR immunization in CFA performed 9 wk earlier. CD8⁺ frequency was reduced in the spleens of EAMG animals ($19.2 \pm 5.1\%$ versus $31.2 \pm 6.5\%$ in controls; $p < 0.05$). No other significant differences in any of these subsets were found between EAMG and healthy rats, both in the spleens and in LNs (Table I). Because naturally occurring regulatory Th cells can be distinguished from activated conventional CD4⁺ Th cells by their high level of CD25 expression (7), we also focused our analysis on the CD4⁺CD25ʰⁱᵍʰ subset; again, no differences were observed in the frequency of CD4⁺CD25ʰⁱᵍʰ T cells in the spleens and LNs of EAMG compared with healthy rats (Table I). We also evaluated LNs from PBS/CFA-, OVA/CFA-, and AChR/CFA-primed animals. Mean cell recovery ranged from 70×10^6 to 114×10^6 cells, and the frequencies of CD4⁺CD25⁺ were: $5.57 \pm 0.59\%$ (SE) in PBS/CFA, $4.78 \pm 0.47\%$ (SE) in OVA/CFA, and $5.56 \pm 0.40\%$ (SE) in AChR/CFA animals, not different from values reported in Table I. The percentage of splenic and LN CD4⁺CD25⁺ and CD4⁺CD25ʰⁱᵍʰ cells overlapped with those reported by Plain and colleagues (28).

Molecular profile of CD4⁺CD25⁺ and CD4⁺CD25⁻ cells isolated from spleens of EAMG and healthy rats

The observation that frequencies of T cell subsets in secondary lymphoid organs (spleens and LNs) of EAMG rats were similar to those of controls, especially the CD25⁺ subset, prompted us to verify if naturally occurring CD4⁺CD25⁺ cells isolated from spleens of healthy and EAMG rats were indeed able to exert regulatory effects on AChR-specific T lymphocyte responses. By means of a two-step purification approach (using anti-CD4 followed

Table I. *Frequency of T cell subsets in secondary lymphoid organs of EAMG and naive rats*

	Spleens		LNs	
	Healthy ($n = 5$)	EAMG[a] ($n = 6$)	Healthy ($n = 5$)	EAMG ($n = 6$)
CD4⁺	35.6 ± 7.9	40.6 ± 4.7	59.2 ± 7.0	56.8 ± 8.7
CD4⁺CD25⁺	4.9 ± 0.8	4.9 ± 0.7	5.1 ± 1.9	4.9 ± 0.6
CD4⁺CD25ʰⁱᵍʰ	1.6 ± 0.4	2.4 ± 1.2	2.1 ± 0.8	2.2 ± 0.9
CD8⁺	31.2 ± 6.5	19.2 ± 5.1[b]	21.5 ± 4.4	22.2 ± 8.7
CD8⁺CD25⁺	2.9 ± 1.8	2.6 ± 0.6	1.8 ± 0.9	0.3 ± 0.2

Data are expressed as mean percent ± SD.
[a]Clinical score of EAMG rats was 2.5 ± 0.6.
[b]EAMG versus healthy rats; $p < 0.05$.
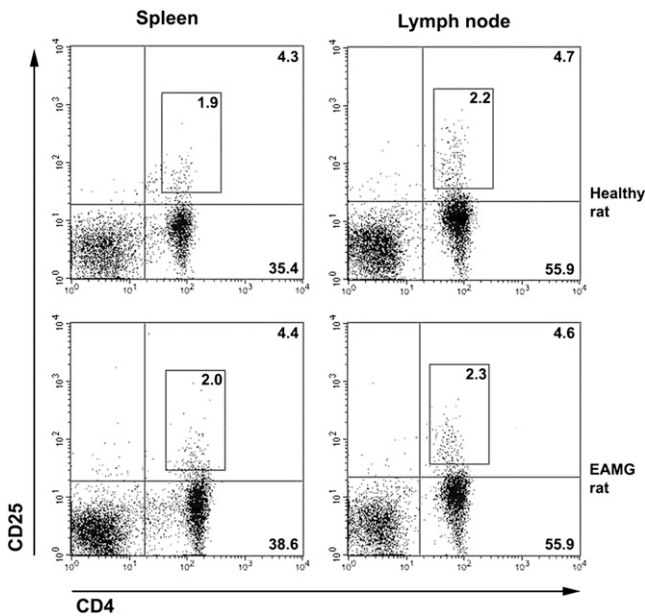
FIGURE 1. Expression of CD25$^+$ on peripheral CD4$^+$ T cells in adult rats. Mononuclear cell suspensions from the spleens (*left panels*) or draining LNs (*right panels*) of a 15-wk-old healthy (*upper panels*) and EAMG (*lower panels*) rats were double stained with mAbs to CD4 and CD25 and analyzed by flow cytometer. Numbers in the corners are the percentage of cells in each gate (i.e., CD4$^+$CD25$^+$, CD4$^+$CD25^{high}, and CD4$^+$CD25$^-$ cells). The plots are representative of typical FACS analysis obtained from EAMG ($n = 6$; mean clinical score 2.5 ± 0.6) and healthy ($n = 5$) rats. Dead cells were excluded by gating on forward and side-scatter channels.

by anti-CD25 Abs; see *Materials and Methods*), we isolated CD4$^+$ CD25$^+$ cells from spleens of EAMG ($n = 6$) and healthy animals ($n = 5$) to be further characterized for purity by FACS analysis,

for transcriptional analysis by qPCR, and for regulatory properties by in vitro coculture experiments. Immunomagnetic purification yielded 88–95% pure CD4$^+$CD25$^+$ lymphocytes. We did not observe any difference in cell recovery between EAMG and healthy rats. Splenic CD4$^+$CD25$^+$ cells (30,000 cells/well) from healthy and EAMG rats never proliferated when challenged in vitro with TAChR or peptide R97–116 [corresponding to an immunodominant T cell epitope from the rat (self) AChR α-subunit] in conventional T cell proliferation assay with irradiated APCs as feeder cells (not shown).

Total RNA was extracted from CD4$^+$CD25$^+$ cells and CD4$^+$ CD25$^-$ cells as control from EAMG and healthy rats. qPCR analysis revealed highly restricted expression of FoxP3 in the CD4$^+$CD25$^+$ cells as compared with the CD4$^+$CD25$^-$ (effector) T cell subset both in healthy and EAMG rats. Transcription of FoxP3 was reduced in CD4$^+$CD25$^+$ cells from EAMG compared with healthy rats (mean $2^{-\Delta Ct} \times 100$ ± SE, 2.89 ± 0.26 EAMG versus 4.80 ± 0.44 healthy rats; $p < 0.05$) (Fig. 2A). The analysis of FoxP3 expression by FACS in healthy and EAMG rats also indicates a reduction in this subset: CD25$^+$FoxP3$^+$ were 4.06 ± 0.10% (SE) in normal rats and 3.15 ± 0.24% (SE) in EAMG animals ($p = 0.025$). The frequency of FoxP3-expressing cells in immunomagnetically separated CD4$^+$CD25$^+$ cells from the spleens of normal animals (88.91 ± 3.10% SE) was not statistically different from that of CD4$^+$CD25$^+$ cells isolated from the spleens of EAMG animals (83.79 ± 1.44% SE). EAMG CD4$^+$ CD25$^+$ cells expressed higher mRNA levels of CTLA4, a functional marker of Tregs (mean $2^{-\Delta Ct} \times 100$ ± SE, 1.59 ± 0.39 EAMG versus 0.82 ± 0.10 healthy rats; $p < 0.05$; Fig. 2B). IFN-γ, a proinflammatory cytokine, was increased in CD4$^+$CD25$^-$ cells from EAMG rats compared with controls (mean $2^{-\Delta Ct} \times 100$ ± SE, 0.46 ± 0.21 EAMG versus 0.16 ± 0.08 control; $p < 0.05$; Fig. 2C). No significant differences in the mRNA levels for IL-6 and IL-10 were noted (Fig. 2D, 2E).
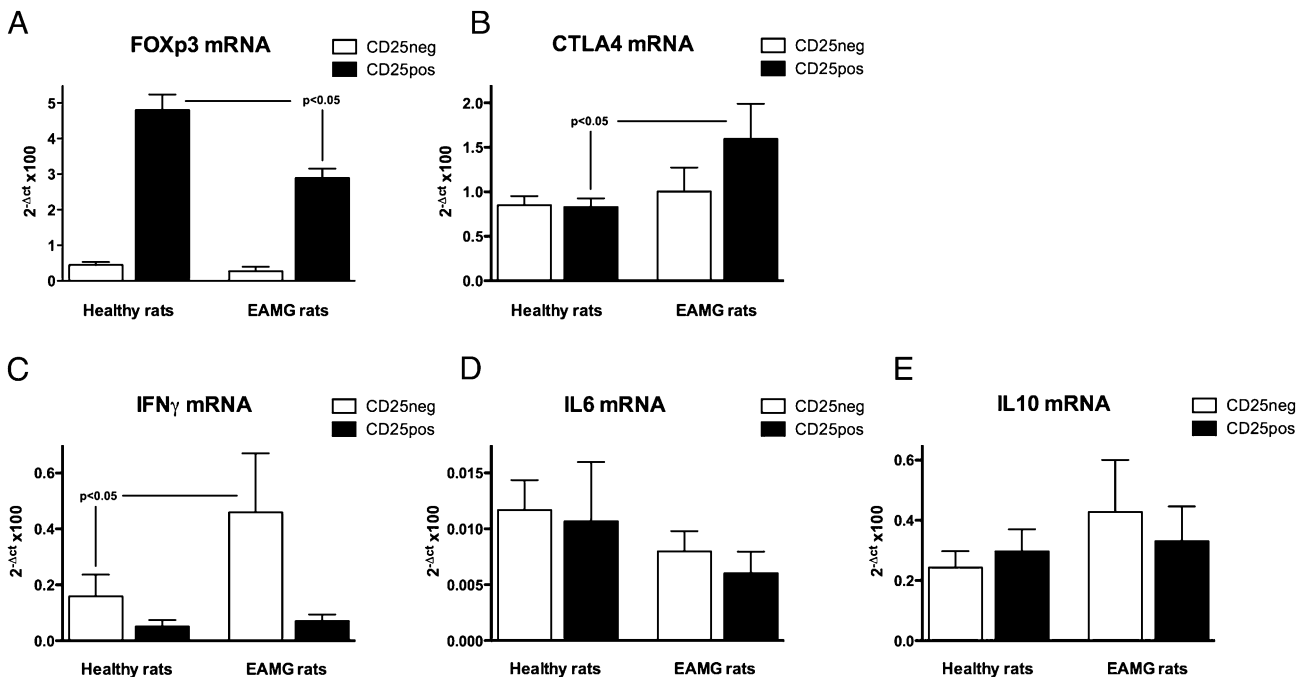
FIGURE 2. Ex vivo real-time qPCR analysis of CD4$^+$CD25$^+$ of CD4$^+$CD25$^-$ cells from healthy and EAMG rats. mRNA expression level of transcription factor FoxP3 and CTLA-4 (*A, B*) and of cytokines IFN-γ, IL-6, and IL-10 (*C–E*) in CD4$^+$CD25$^-$ cells (empty bars) and CD4$^+$CD25$^+$ cells (filled bars) from the spleens of EAMG ($n = 6$) and healthy animals ($n = 5$). Transcript levels were calculated according to the $2^{-\Delta Ct}$ method; actin-β was used as endogenous control to normalize mRNA amount. Bars represent means $2^{-\Delta Ct} \times 100$ + SE. Significant differences between healthy and EAMG animals are indicated as $p < 0.05$.

Naturally occurring CD4$^+$CD25$^+$ cells from naive animals modulate in vitro and ex vivo T cell responses to AChR

The potential regulatory activity of CD4$^+$CD25$^+$ cells isolated from healthy and EAMG rats was initially assessed by conventional coculture experiments in vitro. Purified CD4$^+$CD25$^+$ cells (30,000 cells/well in the presence of 200,000 APCs/well) from the spleens of healthy and EAMG rats did not proliferate in response to TAChR or ConA in vitro, thus showing an anergic phenotype (data not shown). To evaluate their in vitro regulatory properties (29), a rat CD4$^+$ T cell line specific for the immunodominant peptide R97–116 of the rat AChR α-subunit was cocultured with CD4$^+$CD25$^+$ cells purified from the spleens of healthy or EAMG rats. Different doses of CD4$^+$CD25$^+$ cells were used (1:1, 0.2:1, and 0.1:1 CD4$^+$CD25$^+$ cells/R97–116-specific line T cells); mean percentages (± SE) of three separate experiments are shown in Fig. 3A and 3B. CD4$^+$CD25$^+$ cells from healthy rats (Fig. 3A, 3B, filled squares) were able to suppress ConA- (2 μg/ml) and R97–116-induced (5 μg/ml) proliferative responses. Also, CD4$^+$CD25$^+$ cells from EAMG rats showed a similar dose-dependent suppressive activity, but with an overall reduced extent, particularly at the lowest cell ratios (0.2:1 and 0.1:1). Suppression of proliferative responses (expressed as percentage of maximal response of effector cells) by CD4$^+$CD25$^+$ cells from healthy rats at a 0.2:1 ratio was 36.2 ± 8.4% for ConA-induced responses (Fig. 3A) and 56.7 ± 4.6% for R97–116-induced responses (Fig. 3B). CD4$^+$CD25$^+$ cells from EAMG rats modulated the proliferative response to a lesser extent (mean ± SE, 57.1 ± 6.9% and 85.9 ± 7.3% for ConA- and R97–116–induced responses, respectively; $p < 0.05$). Our results demonstrate that CD4$^+$CD25$^+$ cells from

healthy rats exerted inhibitory properties, being able to actively suppress in vitro proliferative responses, whereas CD4$^+$CD25$^+$ cells from myasthenic rats were less effective in suppressing proliferation of Teffs.

As a further control, we also characterized CD4$^+$CD25$^+$ cells from OVA-immunized rats (100 μg OVA in CFA plus 1 mg/rat H37Ra). Cell frequencies were not different from those observed in control nonimmunized rats. Immunomagnetically separated CD4$^+$CD25$^+$ cells were cocultured at a 1:1 cell ratio with the R97–116-specific line T cells to assess their efficacy on the proliferative responses to ConA and R97–116 peptide. OVA-CD4$^+$CD25$^+$ cells were able to reduce the response of the T cell line by 41.8% (when challenged with ConA) and by 51.2% (when challenged with peptide R97–116). Regulatory properties of CD4$^+$CD25$^+$ from OVA-immunized rats were intermediate to those obtained from healthy rats and EAMG rats. Next, we evaluated the ability of CD4$^+$CD25$^+$ cells to modulate ex vivo LNC proliferation to the native AChR, focusing our attention to CD4$^+$CD25$^+$ cells from the spleens of healthy rats. LNs were aseptically removed from TAChR-immunized rats, and single-cell suspensions were challenged in vitro with TAChR (1.25 and 0.25 μg/ml) and ConA (2 μg/ml, not shown) in normal culture conditions and in the presence of CD4$^+$CD25$^+$ cells at a 0.2:1 coculture ratio (40,000 CD4$^+$CD25$^+$ cells and 200,000 LNCs). CD4$^+$CD25$^-$ cells were used as negative control for cocultures (Fig. 3C). We observed that CD4$^+$CD25$^+$ cells from healthy rats were able to reduce TAChR-specific proliferative responses of LNCs at both concentrations tested, with a mean reduction in cell proliferation (± SE) of 74.2 ± 3.2% (TAChR 1.25 μg/ml; $n = 4$) and 73.3 ± 8.2% (TAChR 0.25 μg/ml; $n = 4$) and
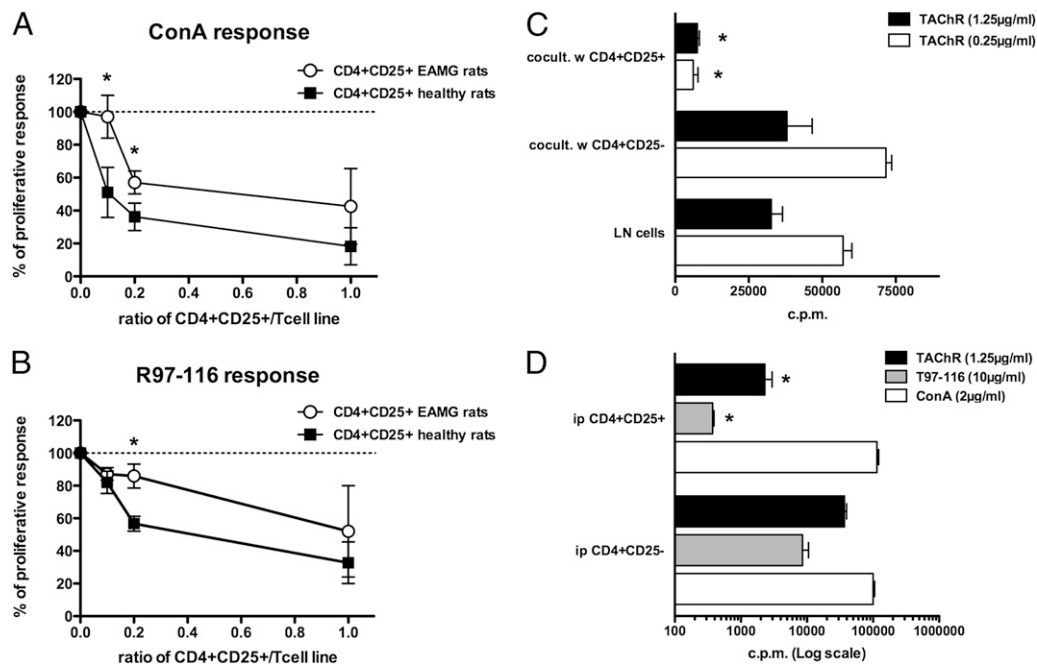
FIGURE 3. CD4$^+$CD25$^+$ cells from healthy but not EAMG rats suppress in vitro and ex vivo proliferative responses to AChR. *A* and *B*, A T cell line specific for peptide R97–116 of the rat AChR α-subunit was stimulated in the presence of different doses (1:1, 0.2:1, and 0.1:1 CD4$^+$CD25$^+$/T cell line) of CD4$^+$CD25$^+$ cells from healthy or EAMG rats. Mean percentages (± SE) of three separate experiments are shown. CD4$^+$CD25$^+$ cells from healthy rats (filled squares) were able to suppress the proliferation of the rat T cell line in a dose-dependent manner (*A*, ConA response, 2 μg/ml; *B*, R97–116 response, 5 μg/ml). Also, CD4$^+$CD25$^+$ cells from EAMG rats showed a similar dose-dependent suppressive activity, showing an overall reduced suppressive capacity, particularly at 0.2:1 cell ratio. *C*, LNCs from TAChR-immunized Lewis rats were challenged with TAChR (1.25 and 0.25 μg/ml) in the presence of CD4$^+$CD25$^+$ cells from healthy rats (CD4$^+$CD25$^+$/LNC coculture ratio 0.2:1). CD4$^+$CD25$^-$ T cells were used as negative control. *D*, LNCs derived from TAChR-immunized rats treated with CD4$^+$CD25$^+$ or CD4$^+$CD25$^-$ cells (2 × 10^6 cells i.p., 24 h postinfection) were challenged in vitro with ConA (empty bars), T97–116 (gray bars) peptide, or TAChR (filled bars). A single administration of CD4$^+$CD25$^+$ cells was able to reduce proliferative responses to TAChR or the immunodominant peptide T97–116 from AChR α-subunit. Results are expressed as mean cpm + SE from triplicate wells and are representative of two to four independent experiments. *$p < 0.05$.

ConA-induced proliferative responses (mean reduction 65.4 ± 3.3%). CD4+CD25− cells, used as controls, did not affect or even increase the proliferative responses of LNCs (Fig. 3C). We also evaluated the ability of CD4+CD25+ from EAMG animals to modulate LNCs response to TAChR in coculture experiments (data not shown); again, the immunoregulatory activity of CD4+CD25+ cells was lower than that exerted by naive CD4+CD25+ cells (mean reduction in cell proliferation ± SE, 30.3 ± 14.4% in the presence of 0.25 μg/ml TAChR).

Administration of CD4+CD25+ cells from naive rats modulates the in vitro antigenic response of T. californica AChR-immunized animals

We tested the potential of CD4+CD25+ to affect the primary response to TAChR in primed rats. Splenic CD4+CD25+ cells isolated from healthy rats were administered i.p. into recipient TAChR-primed rats (2–2.5 × 10⁶/rat) 24 h postimmunization. Control animals received CD4+CD25− cells. Draining LNs were aseptically removed 10 d postimmunization, and LNCs were challenged in vitro with TAChR (1.25 μg/ml), R97–116 or T97–116 peptides (10 μg/ml), and ConA (2 μg/ml). Treatment with CD4+CD25+ cells significantly modulated LNCs specific proliferation to TAChR and to its immunodominant epitope (T97–116 peptide) (Fig. 3D); mean reduction (± SE) of LNCs responses was 77.6 ± 17.2% to TAChR and 94.6 ± 0.1% to T97–116 (n = 4). ConA proliferative responses were not affected by CD4+CD25+ treatment. TAChR-immunized animals did not respond to the self-epitope R97–116 peptide to which rats are normally tolerant (29).

Administration of CD4+CD25+ cells prevents EAMG induction

Our observations suggested we evaluate the ability of CD4+CD25+ cells from healthy rats to modulate EAMG induction in a preventive treatment protocol. EAMG was induced in Lewis rats by a single immunization with 50 μg TAChR purified from T. californica electroplax in CFA. One week postimmunization, rats received the first treatment after being randomly assigned to the CD4+CD25+ group (2–2.5 × 10⁶ CD4+CD25+ cells i.p. once weekly four times) or to the vehicle group (sterile saline i.p. once weekly four times). EAMG symptoms were scored twice weekly, until the end of each experiment, by evaluation of body weight growth and muscular strength. Results from a representative experiment are shown in Fig. 4. Rats that received CD4+CD25+ cells (Fig. 4, filled circles; n = 8) developed a significantly less severe disease compared with vehicle-treated animals (Fig. 4, empty squares; n = 8); at the end of the experiment, the mean weight ± SE (Fig. 4, left panel) was 193.8 ± 12.1 g in the CD4+CD25+ -treated group compared with 151.0 ± 19.9 g (vehicle group); the mean clinical score ± SE (Fig. 4, right panel) was 1.4 ± 0.2

(CD4+CD25+ group) compared with 3.4 ± 0.6 (vehicle group). Protection from the disease persisted until the end of the observation period.

CD4+CD25+ cell treatment does not improve ongoing EAMG

We then assessed the therapeutic potential of CD4+CD25+ cells to improve the course of ongoing EAMG. We adopted a therapeutic protocol of four administrations of CD4+CD25+ cells (2–2.5 × 10⁶ cells i.p. once weekly; n = 8; Fig. 5, filled circles) starting 4 wk postimmunization, when EAMG symptoms became evident. The vehicle group (n = 8; Fig. 5, empty squares) received sterile saline solution i.p. once weekly four times. EAMG rats were scored twice weekly, until the end of each experiment, for gain of body weight and muscular strength. Rats treated with CD4+CD25+ cells developed comparable weakness as the vehicle group (Fig. 5), showing little differences in body weight (Fig. 5, left panel; CD4+CD25+ group: 181.8 ± 6.7 g; vehicle group: 173.3 ± 6.0 g) and in clinical score (Fig. 5, right panel; CD4+CD25+ group: 2.1 ± 0.5; vehicle group: 2.5 ± 0.4) at the end of the experiment.

We have also evaluated whether the failure of our protocol to modulate disease progression derived from insufficient number of CD4+CD25+ cells. Hence, the therapeutic protocol was tested by treating EAMG rats with 4 × 10⁶ CD4+CD25+ cells, with 2 × 10⁶ CD4+CD25+ cells, and with vehicle (n = 18). Moreover, animals received five weekly i.p. treatments, and animal evaluation lasted for 4 wk after the last treatment. Despite the use of more than twice the number of CD4+CD25+ T cells (total cells administered was 20 × 10⁶ compared with 8 × 10⁶ in previous experiments), we did not observe any improvement of EAMG in treated animals, as assessed by clinical score and by evaluation of the anti-rat AChR Abs titer (Supplemental Fig. 1).

Muscle AChR content and anti-AChR Abs are affected by preventive but not therapeutic administration of CD4+CD25+

The efficacy of CD4+CD25+ preventive treatment (and the lack of disease modulation of the therapeutic protocol) was confirmed by evaluating AChR content in skeletal muscles (Fig. 6) and pathogenic Abs (IgG type) against rat- and TAChR (preventive treatment, Fig. 7; therapeutic treatment, Fig. 8). Skeletal muscles and sera were collected at the end of each experiment and stored at −70°C pending assays. EAMG rats receiving CD4+CD25+ preventive treatment had significantly increased muscle AChR content (fmol/g muscle ± SE, 150.1 ± 8.7, n = 8; Fig. 6A, filled bar) compared with vehicle group (fmol/g muscle ± SE, 91.9 ± 11.3, n = 8; Fig. 6A, empty bar; p < 0.05). No differences were observed in EAMG rats receiving therapeutic CD4+CD25+ treatment (Fig. 6B).
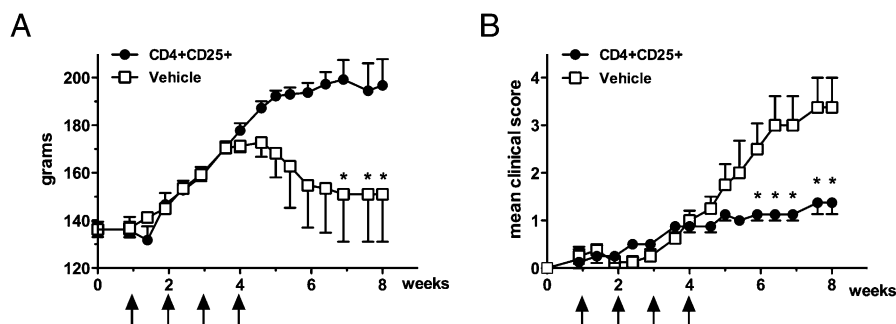
FIGURE 4. Clinical evaluations of preventive treatment with CD4+CD25+ cells. Changes in body weights (A) and clinical scores (B) were recorded in EAMG rats twice weekly at the beginning of experiment until week 8. One week after TAChR immunization, experimental animals were randomly assigned to the CD4+CD25+ group (filled circles, n = 8) or vehicle group (empty squares, n = 8). The arrows indicate the time of treatment administration. The results are representative of three independent experiments. Significant differences in body weight and clinical score are indicated by *p < 0.05.

FIGURE 5. Clinical evaluations of therapeutic CD4+CD25+ cell treatments. Variations in body weight (*A*) and clinical scores (*B*) were recorded in EAMG rats twice weekly at the beginning of experiment until day 67. All animals were TAChR immunized and followed for 4 wk (empty circles) until clinical EAMG manifestations were evident. Animals were then randomly assigned to CD4+CD25+ (filled circles) or vehicle (empty squares) treatment groups. The results are representative of two independent experiments. The arrows indicate the time of the four i.p. administrations. No significant differences were observed.
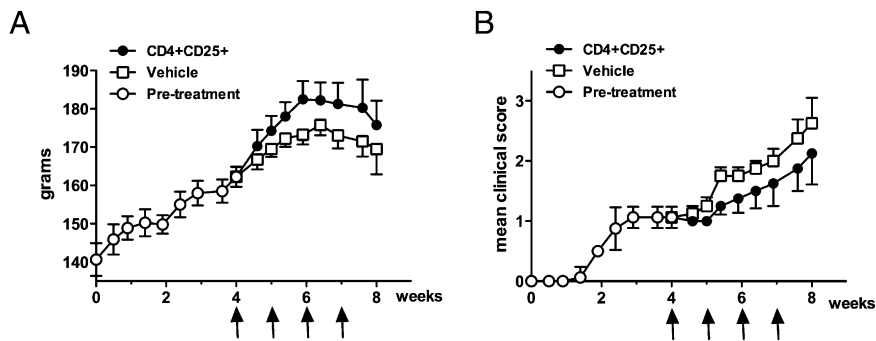
Anti-rat AChR and anti-TAChR Abs titers were significantly reduced in EAMG animals receiving preventive treatment. Fig. 7 shows that IgG anti-rat AChR (Fig. 7*A*; IgG detected by conventional radioimmunological assay, data expressed as pmol/ml serum ± SE) and total anti-TAChR IgG (Fig. 7*B*; total IgG detected by conventional ELISA assay, data expressed as OD_{450} nm ± SE) were significantly reduced compared with the vehicle-treated group (anti-rAChR: treated group 10.7 ± 2.9 versus control 48.3 ± 10.0 pmol/ml, $p < 0.05$; anti-TAChR IgG: treated group 0.34 ± 0.02 versus control 0.42 ± 0.02 OD_{450} nm, $p < 0.05$). We also observed a significant decrease of the levels of the complement-fixing IgG2b and IgG2c isotype (Fig. 7*E*, 7*F*); no differences in the other IgG subtypes were recorded (Fig. 7*C*, IgG1; Fig. 7*D*, IgG2a). These data, in combination with AChR content, confirm the clinical evaluation of treated EAMG animals, and further support the efficacy of preventive administration of CD4+ CD25+ cells.

A significant reduction of total anti-TAChR IgG and IgG2b subtype was also detected in EAMG rats receiving the therapeutic treatment (Fig. 8*B*, 8*E*), but no modification in the level of

FIGURE 6. Muscle AChR content in EAMG rats following preventive or therapeutic CD4+CD25+ treatments. AChR content in rat skeletal muscles was assessed by conventional binding assay with [125I]anti-BTX. Preventive treatment (*A*) was able to prevent AChR loss, as compared with vehicle-treated EAMG rats. No differences were observed after therapeutic treatment (*B*). AChR content in muscle of healthy rats was 186.7 ± 20.3 fmol/g muscle. Data are expressed as femtomoles of AChR binding sites per gram of rat muscle ± SE. *$p < 0.05$.

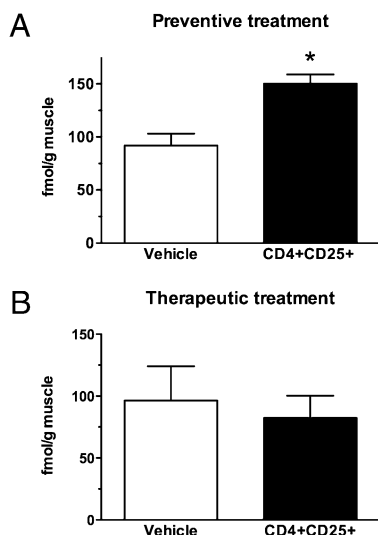pathogenic anti-rat AChR IgG Ab was measured (Fig. 8*A*) or in other IgG subclasses.

Preventive treatment affects the proliferative response to T. californica *AChR*

Draining LNs were removed and processed to single-cell suspension to assess proliferative responses to TAChR (1.25 and 0.25 μg/ml) and ConA (2 μg/ml) (Fig. 9*A–C* for preventive treatment and Fig. 9*D–F* for therapeutic treatment). A strong modulation of LNC response to TAChR at both concentrations used was observed in EAMG animals receiving the preventive administration of CD4+CD25+. Proliferative responses to TAChR (1.25 μg/ml) were 6299 ± 1690 mean cpm ± SE in CD4+CD25+-treated EAMG and 43,786 ± 16,654 mean cpm ± SE in vehicle-treated EAMG (Fig. 9*B*; $p < 0.05$); responses to TAChR (0.25 μg/ml) were 4793 ± 1513 mean cpm ± SE in CD4+CD25+-treated EAMG and 14,101 ± 5615 mean cpm ± SE in vehicle-treated EAMG (Fig. 9*C*; $p < 0.05$). Modulation of LNC response to TAChR, but only at the lowest concentration used, was observed in EAMG animals treated after disease onset (Fig. 9*F*, therapeutic schedule); no modulation of ConA-stimulated T cell response was observed (Fig. 9*A*, 9*D*).

Ex vivo analysis from preventive CD4+CD25+ treated rats shows Th1/Th2 shift

By means of ex vivo qPCR on snap-frozen spleens and LNs, we investigated mRNA expression levels of the typical functional markers of Th1 cell subset (IFN-γ), Th2 cell subset (IL-6 and IL-10), and CD4+CD25+ regulatory cells (FoxP3 and CTLA-4) (Fig. 10). Preventive CD4+CD25+ treatment induced an increase of the mRNA levels of FoxP3 (Fig. 10*A*, mean $2^{-\Delta Ct} \times 100$ ± SE, 0.932 ± 0.110 treated versus 0.325 ± 0.056 vehicle; $p < 0.05$) and CTLA-4 (Fig. 10*B*, mean $2^{-\Delta Ct} \times 100$ ± SE, 2.396 ± 0.302 treated versus 1.356 ± 0.121 vehicle; $p < 0.05$). Therapeutic treatment did not induce modification of FoxP3 transcription (Fig. 10*F*), but only CTLA-4 was found increased (Fig. 10*G*, mean $2^{-\Delta Ct} \times 100$ ± SE, 3.706 ± 0.029 treated versus 2.977 ± 0.45 vehicle; $p < 0.05$).

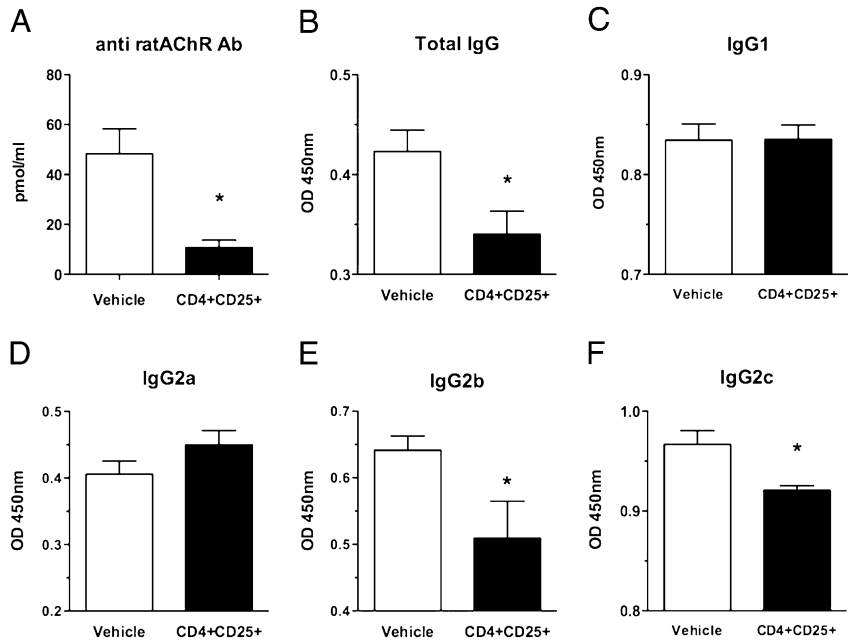CD4+CD25+ preventive treatment induced a reduction in IFN-γ mRNA expression level (Fig. 10*C*, mean $2^{-\Delta Ct} \times 100$ ± SE, 0.091 ± 0.015 treated versus 0.260 ± 0.096 vehicle; $p < 0.05$) and in IL-10 mRNA expression level (Fig. 10*D*, mean $2^{-\Delta Ct} \times 100$ ± SE, 0.086 ± 0.013 treated versus 0.165 ± 0.039 vehicle; $p < 0.05$). Only IL-10 (Fig. 10*I*, mean $2^{-\Delta Ct} \times 100$ ± SE, 0.083 ± 0.036 treated versus 0.201 ± 0.011 vehicle; $p < 0.05$) but not IFN-γ mRNA (Fig. 10*H*) was found significantly decreased by therapeutic treatment. IL-6 transcripts were unchanged in both preventive and therapeutic CD4+CD25+ treatments (Fig. 10*E*, 10*J*). Parallel analyses in the spleens from EAMG animals did not reveal

FIGURE 7. Anti-rat AChR and anti-TAChR Abs after preventive CD4+CD25+ treatments. Pathogenic anti-rat AChR Abs (*A*) as well as anti-TAChR Abs (*B–F*) were evaluated in serum samples collected at the end of the experiments ($w = 8$) by conventional radioimmunological assay (for rAChR) and ELISA. Anti-rat IgG is expressed as picomoles per milliliter rat serum (mean ± SE); total IgG and IgG subclass titers for anti-TAChR are expressed as mean OD at 450 nm ± SE. *$p < 0.05$.

any significant changes independent of any of the two treatments (not shown).

Frequencies of FoxP3+ cells in the LNCs were assessed by FACS, and values were 3.15 ± 0.64% (SE) in the vehicle, 3.40 ± 1.30% (SE) in the preventive treatment group, and 2.95 ± 1.18% (SE) in therapeutic treatment group. A modest, albeit not statistically significant, increase was observed in the preventive treatment group. Real-time PCR analysis and cytokine production assay of TAChR stimulated LNCs from CD4+CD25+-treated EAMG rats according to the preventive and therapeutic schedule. We also analyzed IFN-γ, IL-6, and IL-10 cytokine production in the culture supernatant from TAChR-stimulated LNCs, in parallel with qPCR; results are summarized in Supplemental Fig. 2.

Discussion

Autoimmunity is a consequence of an unbalance between immune sensitization toward self-Ags and immune regulatory processes that should control effector mechanisms from inducing immune pathologies (30, 31). Accumulating evidence indicates that CD4+ CD25+ Tregs are crucial in maintaining self-tolerance and inhibiting the development of autoimmunity. Treg function and distribution in lymphoid tissue has been studied in autoimmune diseases and in the corresponding models (14, 32–34), and experimental settings analyzed so far tested Tregs for their altered activity in autoimmune diseases (35). Recent data indicate also that administration of CD4+CD25+ Tregs improves ongoing experimental autoimmune diseases (10, 36). Sakaguchi and colleagues (6) proposed
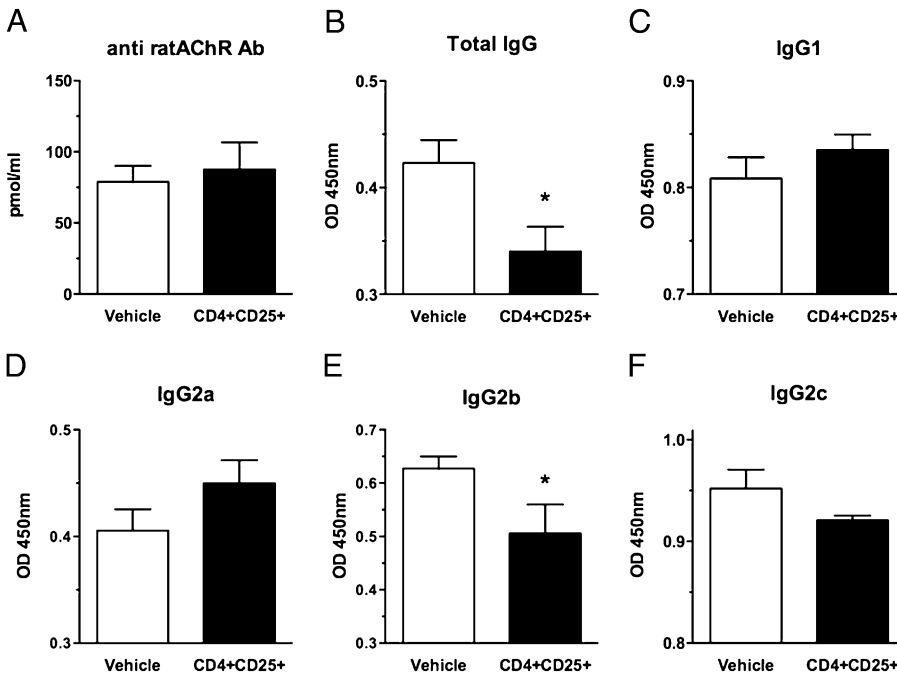
FIGURE 8. Autoantibodies production after therapeutic CD4+CD25+ cell treatments. Pathogenic anti-rat AChR Abs (*A*) as well as anti-TAChR Abs (*B–F*) were evaluated in serum samples collected at the end of the experiments ($w = 8$) by conventional radioimmunological assay (for rAChR Abs) and ELISA (for TAChR Abs). Anti-rat IgG is expressed as picomoles per milliliter rat serum (mean ± SE); total IgG and IgG subclass titers for anti-TAChR are expressed as mean OD at 450 nm ± SE. *$p < 0.05$.
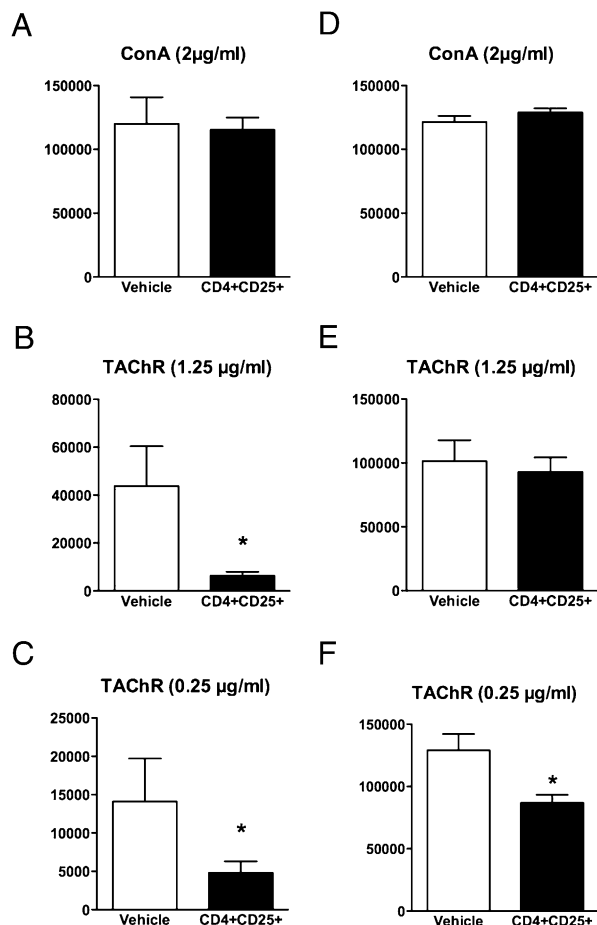
FIGURE 9. Ex vivo proliferation of LNCs in response to TAChR after preventive and therapeutic treatments. Proliferative responses of LNCs from CD4⁺CD25⁺ cells (filled bars) or vehicle-treated (empty bars) rats collected at the end of the experiments ($w = 8$) and challenged in vitro with TAChR and ConA. *A–C* show LNC responses after preventive CD4⁺ CD25⁺ treatment and *D–F* after therapeutic treatment. Data are expressed as mean cpm $\pm$ SE. $*p < 0.05$.

the CD25 molecule (the IL-2R α-chain) as a phenotypical marker of regulatory cells, and this Ag still remains instrumental in identifying and selecting Tregs from a heterogeneous cell population (37). However, the most accurate definition of Tregs came with the identification of the FoxP3 (38), allowing more precise characterization of the CD4⁺CD25⁺ regulatory population.

These observations were the basis for our investigation on the function of naturally occurring CD4⁺CD25⁺ cells in healthy and EAMG rats. We focused our study on naturally occurring CD4⁺ CD25⁺ cells from naive animals to verify whether this subset was indeed capable to protect rats from the development of an autoimmune response via a cross-reaction between TAChR and rat (self) AChR. Moreover, we also assessed their potential use in restoring tolerance to AChR in ongoing EAMG.

The results of our study demonstrated that 1) no differences were found in the frequency of LN and splenic CD4⁺CD25⁺ subsets between EAMG and control animals 2) CD4⁺CD25⁺ cells from EAMG rats have an altered transcription profile of FoxP3 and CTLA-4 compared with normal animals; 3) CD4⁺CD25⁺ cells from normal rats significantly suppress in vitro proliferative responses to AChR; 4) preventive treatment of EAMG with CD4⁺ CD25⁺ cells is effective in modulating disease manifestations; and 5) therapeutic treatment fails to modulate ongoing EAMG.
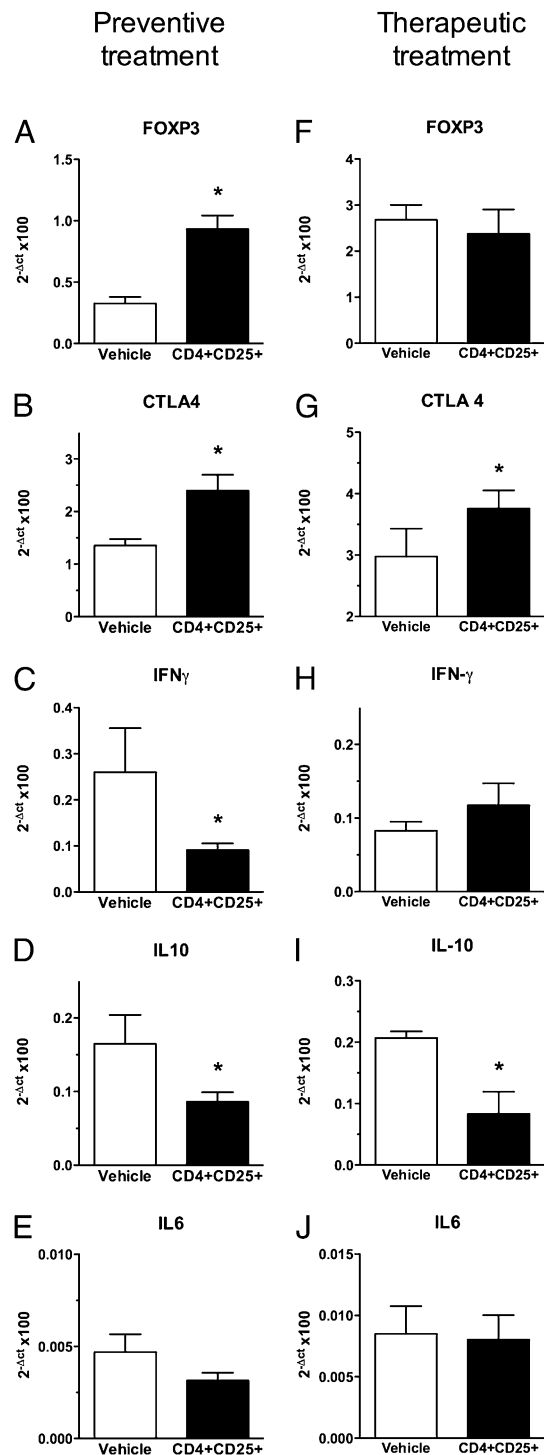
FIGURE 10. Ex vivo real-time PCR analysis of LNCs from CD4⁺ CD25⁺-treated EAMG rats according to the preventive and therapeutic schedule. mRNA expression level of transcription factor FoxP3, CTLA-4 (markers of CD4⁺CD25⁺ cells), IFN-γ (Th1-type cytokine), IL-10, and IL-6 (Th2-type cytokines) in LNCs from vehicle- (empty bars) and CD4⁺ CD25⁺-treated (black bars) rats collected at the end of the experiments ($w = 8$). *A–E* correspond to the preventive treatment and *F–J* to therapeutic treatment of EAMG rats. Transcript levels were calculated according to the $2^{-\Delta Ct}$ method; actin-β was used as endogenous control to normalize mRNA amount. Bars represent means $2^{-\Delta Ct} \times 100 \pm$ SE. $*p < 0.05$.

FACS analysis of CD4⁺CD25⁺ Tregs in spleen and LNs of EAMG compared with naive rats showed no differences in the percentage of both CD4⁺CD25⁺ T cells and CD4⁺CD25^high T cells.

Aricha and colleagues (10) found a reduced number of Tregs in EAMG compared with healthy Lewis rats, but they focused their study on circulating peripheral blood lymphocytes. Our data show that CD4$^+$CD25$^+$ cells are present at similar frequencies in secondary lymphoid organs of normal and EAMG rats (Table I), suggesting the possibility that their regulatory functions might be altered in EAMG.

We restricted our analysis to the CD25 marker, because this Ag is currently available for purification of naturally occurring Tregs using immunomagnetic separation techniques and sterile conditions necessary for subsequent in vivo studies. Naturally occurring Tregs have been isolated from the spleen and not from LNs of healthy animals due to the low number of cells that can be obtained from the latter lymphoid organ, not adequate to allow in vivo studies. FoxP3 mRNA was restricted to CD4$^+$CD25$^+$ cells only; decreased FoxP3 and increased CTLA-4 transcripts in EAMG naturally occurring regulatory cells were found as compared with CD4$^+$CD25$^+$ cells from naive animals. The different transcriptional profile of CD4$^+$CD25$^+$ cells from normal and EAMG animals (Fig. 2) supported the use of naive CD4$^+$CD25$^+$ cells for EAMG treatment.

The role of CTLA-4 has been extensively studied in mice through the last decade and provides a Treg-Teff mechanism valid in those animals. However, little is known about the CD4$^+$CD25$^+$ subset in other species, such as rats, as also pointed out by Holm et al. (39): their study describes the phenotype of rat CD4$^+$CD25$^+$ FoxP3$^+$ T cells and the site in which these cells exert regulation in an autoimmune diabetes model. They state also that several mRNAs are upregulated in CD4$^+$CD25$^+$ T cells compared with CD4$^+$CD25$^-$ T cells, including FoxP3, Lag-3, CD80, IL-10, and CTLA-4. However, their results on the latter molecule are not conclusive, these analyses having been performed only at the mRNA level. Balandina et al. (16) tried to address the possible role of the surface and intracellular CTLA-4 molecule in the regulatory function of CD4$^+$CD25$^+$ thymocytes from patients with MG. However, they stated that no surface expression of CTLA-4 was observed in their studied sample populations, whereas the frequency of CD4$^+$CD25$^+$ thymocytes expressing cytoplasmic CTLA-4 was moderately higher in cells isolated from newborn thymi compared with those obtained from patients with MG and healthy adults; moreover, there was no difference between patients and healthy young adults. Our preliminary FACS analysis data on FoxP3/CTLA-4 expression by spleen cells of normal and EAMG rats (not shown) apparently suggest that frequencies of both FoxP3$^-$/CTLA-4$^+$ and FoxP3$^+$/CTLA-4$^+$ cells are reduced in TAChR-immunized animals. The conclusion that can be drawn from these studies is that the role of CTLA-4 in rats EAMG (and likely in human MG) is controversial and should be investigated further.

More strikingly, the functional analyses by in vitro cocultures confirmed that CD4$^+$CD25$^+$ cells from healthy animals are more effective in modulating in vitro T cell responses to native AChR and its relevant derived immunodominant epitope R97–116 of the rat AChR α-subunit, reported to be an immunodominant and myasthenogenic T cell epitope (29). CD4$^+$CD25$^+$ cells from EAMG rats showed only a modest suppressive activity. Naive CD4$^+$CD25$^-$ cells, used as negative control, did not exert any suppression.

These findings supported the investigation of naturally occurring regulatory CD4$^+$CD25$^+$ cells from healthy rats as a tool to modulate the onset and progression of EAMG. Indeed, our preventive treatment protocol (four administrations starting 7 d after TAChR immunization) modified the course of the disease as shown by the positive trend of clinical scores and serial assessment of body weight in treated rats. On the contrary, our treatment protocol

(starting at the onset of EAMG) was not effective in the control of the immunological response to AChR. A very modest improvement of the clinical score and body weight was seen at the beginning of the treatment, but without any further improvement. We also tested a different treatment protocol and prolonged the animal follow-up for 4 wk after the last CD4$^+$CD25$^+$ administration (as we have done in the preventive treatment). Despite the use of more than twice the number of CD4$^+$CD25$^+$ T cells, we did not observe any improvement of EAMG manifestations in treated animals. All animals developed anti-AChR Abs without statistical differences among treatment groups.

Our protocols differ from that adopted by Aricha et al. (10); their treatment started at disease induction and continued until sacrifice, with a similar amount of cells administered at 2-wk intervals. Their prolonged preventive protocol was effective in preventing and modulating EAMG manifestation. However, their treatment schedule cannot be considered as a therapeutic protocol because it did not start after the onset of clinical manifestations (i.e., at least 4 wk postimmunization). Therefore, there are still open issues regarding the timing, dose, and schedule of administration to confirm the actual therapeutic potential of Tregs in EAMG. In this regard, it must be noted that full-blown EAMG is a severe disease characterized not only by weakness but also by muscle wasting, a feature that might hinder the observation of a clinical effect of Tregs, at least in our hands. Moreover, we preferred not to select animals according to the clinical score (mild versus severe) of the disease for our preliminary studies.

The clinical findings obtained by our preventive protocol were confirmed by in vitro studies on rat muscle AChR content, together with reduced anti-rat AChR Abs and anti-TAChR IgG subclasses as well as proliferative responses to TAChR. None of these changes were induced by the therapeutic protocol.

Several studies tried to address the role of Th1- and Th2-type cytokines in EAMG and their changes induced by different approaches to restore tolerance to AChR (2, 40, 41). In the C57/Bl6 mouse models of EAMG, pathogenic anti-AChR Abs belong to the IgG2a subclass and are associated with Th1-type responses. In rats, the anti-TAChR Ab response is IgG2b dominant in the Lewis strain and IgG1 dominant in Brown-Norway strain (41). However, both strains are susceptible to EAMG, as all IgG subclasses are capable of binding complement and promote degradation of the neuromuscular junction. In rats, IgG2b subclass is IFN-γ dependent. Hence, we performed ex vivo analysis of IFN-γ, IL-6, and IL-10 (as markers for Th1/Th2 subsets) in parallel with FoxP3 and CTLA-4 transcripts in LNs of treated animals. We studied draining LNs for our molecular analysis because they are the major site of accumulation of Tregs (34) and the site where Tregs may exert their inhibitory activity.

The preventive CD4$^+$CD25$^+$ treatment induced a downregulation of the Th1 cytokine IFN-γ and influenced to a lesser extent IL-10 transcription, a marker of the Th2 compartment. These modifications were associated with an increase in FoxP3. The frequencies of FoxP3$^+$ cells in the LNCs were also assessed by FACS, and values were 3.15 ± 0.64% (SE) in the vehicle, 3.40 ± 1.30% (SE) in the preventive, and 2.95 ± 1.18% (SE) in therapeutic groups, although not statistically significant. The increase in CTLA-4 mRNA level was not confirmed by FACS staining. The same analysis was performed in the spleens of treated animals, without detecting any modification in the mRNA transcripts, confirming again that LNs are the major site of the autosensitization to AChR and the place were main regulatory phenomena occur. These results suggested that preventive treatment with spleen-derived CD4$^+$CD25$^+$ cells could result in a series of events, such as 1) migration to draining LNs in vivo and not to spleens; 2) local shift

in the molecular pattern of Th1/Th2 cytokine milieu to a Th2 polarization; 3) control of myasthenogenic Th cell subset expansion; and 4) reduced autoantibody production. EAMG protection in our experiments could also have other alternative explanations (i.e., newly generated Tregs may arise in LNs and other lymphoid tissues posttreatment during adaptive tolerogenic responses), as suggested by other authors (10, 42). These data indicated that preventive CD4+CD25+ treatment is restricted to draining LNCs that are the effector lymphoid organ in our animal model of MG.

The translational therapeutic potential of Tregs depends on their ability to reverse autoimmune responses in an ongoing disease (43). Our experiments showed that therapeutic CD4+CD25+ treatment did not improve ongoing EAMG but also that it did not cause exacerbation of the disease, at least with the two schedules adopted. In this regard, the proliferative response of LNCs derived from CD4+CD25+ treated animals was weakly downregulated, and the cytokine milieu in LNCs was partially affected by the treatment, with upregulation of CTLA-4 (marker for Tregs) and a downregulation of IL-10. We further investigated the Th1/Th2 shift (Fig. 10) by evaluating IFN-γ, IL-6, and IL-10 mRNA expression and secretion in the culture supernatants from TAChR-stimulated LNCs (Supplemental Fig. 2). Within the limits of our experimental conditions, analysis of the same target(s) at the molecular and protein levels provided data that are in good agreement, thus confirming our observations. We speculate that the incapacity of CD4+CD25+ cells to revert ongoing EAMG may be due to inadequate control of Teffs leading to B cell activation and differentiation into AChR Ab-secreting plasma cells. Indeed, it is possible that the potential regulatory activity of naturally occurring CD4+CD25+ T cells from syngeneic donors is only effective at the early steps of T cell activation to the AChR. On the contrary, CD4+CD25+ T cells will not be able to control the progression of ongoing EAMG in recipient rats, when memory T but also B cells and Ab-secreting plasma cells are already generated. A recent paper by Liu B et al. (44) further addressed the potential use of CD4+CD25+ cells in modulating EAMG in the mouse model. They showed that injection of immune complexes composed of IL-2 and anti–IL-2 mAb induced an expansion of Tregs followed by suppression of autoreactive T and B cell responses to AChR and attenuated EAMG symptoms, providing support to a potential clinical use for treatment of MG. Their findings should be also investigated in the rat EAMG model to provide further support to the role of CD4+CD25+ T cells in controlling disease manifestations.

Our study investigated naturally occurring CD4+CD25+ cells ex vivo isolated from naive animals, and in this context, we are evaluating the efficacy of a polyclonal repertoire of cells against the expansion of Ag-specific T cells (induced by TAChR immunization in CFA). The approach proposed by Aricha et al. (10) used in vitro-expanded effector CD4+ cells in conditions that favor the induction of a regulatory phenotype (stimulation with anti-CD3 and anti-CD28 Abs in the presence of TGF-β and IL-2). Both naturally occurring and ex vivo-generated immunoregulatory cells present a potential translational application for the treatment of MG; however, our study could also contribute to better understanding of the role of naturally occurring cells in the lack of immunological control of the disease, whereas the ex-vivo generation of immunomodulatory cells might represent a more rapid approach to the design of a clinical pilot study in humans. A combined study aiming to compare both preventive approaches (i.e., starting in the early phases of the disease induction) will be of extreme interest. It is noteworthy that both studies were able to demonstrate the potential effect of polyclonal regulatory cells to modulate an Ag-specific immune response and suggest the need for further studies aiming at the improvement of Treg availability,

efficiency, and efficacy as a candidate cellular immunotherapy of MG.

Disclosures
The authors have no financial conflicts of interest.

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