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*J Immunol* 2001; 166:4853-4862; doi: 10.4049/jimmunol.166.8.4853
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Transgenic Expression of IL-10 in T Cells Facilitates Development of Experimental Myasthenia Gravis

Norma S. Ostlie,* Peter I. Karachunski,* Wei Wang,* Cristina Monfardini,* Mitchell Kronenberg,+ and Bianca M. Conti-Fine2,3,†‡

Ab to the acetylcholine receptor (ACHR) cause experimental myasthenia gravis (EMG). Th1 cytokines facilitate EMG, whereas Th2 cytokines might be protective. IL-10 inhibits Th1 responses but facilitates B cell proliferation and Ig production. We examined the role of IL-10 in EMG by using wild-type (WT) C57BL/6 mice and transgenic (TG) C57BL/6 mice that express IL-10 under control of the IL-2 promoter. We immunized the mice with doses of AChR that cause EMG in WT mice or with low doses ineffective at causing EMG in WT mice. After low-dose AChR immunization, WT mice did not develop EMG and had very little anti-AChR serum Ab, which were mainly IgG1, whereas TG mice developed EMG and had higher levels of anti-AChR serum Ab, which were mainly IgG2, in addition to IgG1. At the higher doses, TG mice developed EMG earlier and more frequently than WT mice and had more serum anti-AChR Ab. Both strains had similar relative serum concentrations of anti-AChR IgG subclasses and IgG and complement at the muscle synapses. CD8+−depleted splenocytes from all AChR-immunized mice proliferated in the presence of AChR and recognized a similar epitope repertoire. CD8+−depleted splenocytes from AChR-immunized TG mice stimulated in vitro with AChR secreted significantly more IL-10, but less of the prototypic Th1 cytokine IFN-γ, than those from WT mice. They secreted comparable amounts of IL-4 and slightly but not significantly reduced amounts of IL-2. This suggests that TG mice had reduced activation of anti-Torpedo AChR Th1 cells, but increased anti-AChR Ab synthesis, that likely resulted from IL-10-mediated stimulation of anti-AChR B cells. Thus, EMG development is not strictly dependent on Th1 cell activity. The Journal of Immunology, 2001, 166: 4853–4862.

D4+ T cells comprise Th1 and Th2 cells, which differ in their function and the cytokines they secrete (1–3). Th1 cells have been implicated in the pathogenesis of both T cell-mediated and Ab-mediated autoimmune diseases because they secrete proinflammatory cytokines, can be cytotoxic, and help synthesis of complement-binding IgG subclasses that should be effective in causing tissue damage (1–3). Th2 cells are not cytotoxic and might protect from some autoimmune responses, because they down-regulate the activity of both Th1 cells and APC by secreting antiinflammatory cytokines such as IL-4, IL-5, and IL-10 (1–3). However, Th2 cytokines have a variety of effects on the immune functions that may result in contrasting consequences on normal and autoimmune responses.

Ab against the muscle acetylcholine receptor (MACHR)4 cause myasthenia gravis (MG) and its animal model, experimental MG (EMG) (4). Th1 cells and/or Th1 cytokines are involved in the pathogenesis of MG (5–7) and EMG (8–12), whereas Th2 or TGF-β-secreting cells (sometimes referred to as Th3 cells) may have a protective role (13–20).

IL-10 is an important effector cytokine of Th2 cells and other cell types, including Ly-1 B cells, keratinocytes, activated macrophages, and mast cells. IL-10 has a broad range of activities that include anti-inflammatory activity, costimulation of thymocytes, stimulation of mast cell proliferation, and stimulation of B cells differentiation (1, 2, 21).

To understand the role of IL-10 in EMG, we have examined whether transgenic (TG) expression of IL-10 influences susceptibility to EMG. We have used wild-type (WT) C57BL/6 (B6) mice, and IL-10-TG mutants of B6 background in which the T cell-specific human IL-2 promoter drives the expression of mouse IL-10 transgene (22). The T cells that synthesize TG IL-10 include activated, IL-2-secreting Th1 cells, which produce IL-10 only transiently in response to T cell activation (22). Thus, activated Th1 cells in these mice may down-regulate their own activity, including secretion of TG IL-10. Because of the transient expression of the transgene, the immune system of these mice is not significantly different from the control littermates: serum IgG levels and numbers and phenotype of T and B cells are normal (22). Moreover, although IL-10 is a potent down regulator of Th1 cells (1–3), IFN-γ synthesis is reduced but not eliminated in these TG mice (22).

We have immunized IL-10 TG and WT mice with acetylcholine receptor (ACHR) and examined the appearance of EMG and the characteristics of their anti-AChR Ab and T cell responses.

Materials and Methods

Mice

WT breeders were obtained from The Jackson Laboratory (Bar Harbor, ME). TG breeders had been obtained from eggs of (B6 × C3H)F1 females...
mated to B6 males. Their establishment and characterization were described previously (22). Because the genetic background of mice influences their susceptibility to EMG (4) and the mice used to establish the TG mice were of mixed background, littermates with or without the IL-10 transgene could not be used to investigate the effects of TG expression of IL-10 on susceptibility to EMG. The susceptibility to EMG of B6 mice is well characterized (4). To obtain TG mice suitable for these studies, the IL-10 TG founders were back-crossed with B6 mice six times. Therefore, the genetic background of the resulting TG mice we used was almost entirely B6, and we could compare their susceptibility to EMG with that of WT mice.

Previous studies have characterized the properties of the immune system and immune responses of the TG mice we used (22, 23). They have normal T and B lymphocyte development. Their Th2 activity, as judged by the secretion of IL-4, is similar to the control littermates (22). They secrete more IL-10 than the control littermates but less IFN-γ, indicating that TG IL-10 secretion by the Th1 cells results in a self-limitation of the activity of Th1 cells (22). We bred both strains at the animal facility of the University of Minnesota.

**Purification of Torpedo AChR (TACHR)**

We purified TACHR from electric tissue of *Torpedo californica* as alkali-stripped TACHR-rich membrane fragments (24). The AChR structure is very conserved among evolution: TACHR is highly homologous to mammalian MACHR and suitable to induce EMG (4). We measured the protein concentration by the Lowry assay (25) and the TACHR concentration as α-bungarotoxin (αBTX)-binding sites (24). The TACHR preparation was used containing 3.8–5.8 nmol of sites/mg protein. SDS-PAGE analysis (24) showed that the TACHR preparations contained only the four TACHR subunits as the main protein bands. For cell cultures, we diluted the TACHR-rich membrane fragments in RPMI 1640 as needed and sterilized them by UV irradiation. For immunization and Ab assay, we solubilized the membranes in 1% Triton X-100 (24), diluted them to 0.5 mg/ml in PBS, and stored them at −80°C.

**Peptides**

We determined the epitope repertoire on the AChR α subunit, which dominates the sensitization of anti-TACHR CD4+ T cells in B6 mice (24, 26), by using a panel of synthetic peptides—20 residue long and overlapping by 5 residues—that spanned the sequence of the TACHR α subunit. We reported their characterization previously (24). We used solutions of the individual peptides in PBS sterilized by UV irradiation and stored frozen.

**Immunization**

We immunized 8- to 10-wk-old female mice by s.c. injections along the back and at the base of the tail of solubilized TACHR (2–30 μg in 100 μl of PBS) emulsified with 100 μl of CFA. We boosted them twice at 4-wk intervals with the same amount of TACHR in incomplete CFA, and a third time 5–7 days before sacrifice. We have shown previously that this immunization procedure, when using 20–40 μg of AChR, induces EMG (e.g., Refs. 12, 15, 17, 18, 24, 26, 27). We used female mice because after they reach maturity and until 6 mo of age, their weight is constant, both in the WT and the TG strain, whereas the weight of males increases with age, thus making it impossible to measure their strength with the holding test we used, described below.

**Evaluation of clinical symptoms of EMG**

EMG symptoms in mice may not be obvious, and are difficult to quantify by inspection (4). We quantified the EMG weakness with a forced exercise, sensitized by a small amount of pancuronium bromide (0.03 mg/kg i.p.) just before the test (27). The mice hang from a grid: we measured the time it took them to release the hold and fall three times (holding time). To verify the myasthenic nature of the weakness, we injected i.p. edrophonium chloride (Reversol; Organon, West Orange, NJ). Reversol is a cholinesterase inhibitor and it immediately increased the strength of mice that have EMG. We described this test in detail previously (12, 15, 17, 18, 27). The test is parametric and gives a quantitative and reliable assessment of the severity of the muscle weakness, as verified by the finding that repeated tests of the same mouse yield comparable holding times (e.g., Ref. 12; also see left panels of Fig. 1, A and B). The average holding time of 285 naive WT mice was 11.4 ± 1.5 min (12). Naive TG mice had holding times indistinguishable from WT mice (also, see Ref. 18). We considered myasthenic the mice with holding times of 8.3 min (holding time of normal mice −2 SD) or less. Among EMG mice, we differentiated the severity of the myasthenic weakness as follows: mild EMG, holding times between 8.3 and 6.75 min (holding time of normal mice −3 SD); moderate EMG, holding times between 6.75 and 5.2 min (holding time of normal mice −4 SD); severe EMG, holding times of 5.2 min or less. Mice that were paralyzed or that died of EMG are represented in the figures as having holding time of zero.

We tested each mouse at least once during the week before beginning the immunization treatment. This ensured that we used mice that behaved consistently and that we excluded the few unusually “jittery” animals that would jump from the grid rather than hanging from it. We tested the mice every 1–3 wk, starting on the day before the first immunization. We performed the test without knowledge of the mouse treatment. We used the two-factor ANOVA test with the MacANOVA program (a free, noncommercial, interactive statistical analysis program written by Gary W. Oehlert and Christopher Bingham, School of Statistics, University of Minnesota, Minneapolis-St. Paul, MN) to determine the statistical significance between the holding times over time of TG and WT mice that had received the same TACHR immunization treatment. We used the following model: response = treatment + time + E(mouse) + treatment × time, where E(mouse) was the holding time in minutes, treatment was the presence (or absence) of the IL-10 transgene, time was the time of the individual test (measured as weeks after the beginning of the immunization), and E(mouse) was the degrees of freedom (a function of the number of the mice in the groups analyzed). We considered a difference to be significant when p ≤ 0.05.

**Production of the MACHR content correlates with EMG severity (4).** However, it can be measured only after sacrificing the mice, whereas the holding test permits to follow the time course of the disease. In another study, we verified that a reduction in holding time correlated with reduced MACHR content of the muscle, measured at the time of the clinical observation: for mice with EMG there was a good statistical correlation between holding time and MACHR content.

**Anti-AChR Ab assay**

We obtained sera after each clinical testing. We measured the serum concentration of anti-TACHR Ab by radioimmunoprecipitation assay (RIPA), using TACHR solubilized in Triton X-100 and labeled by 125I-αBTX (23, 25). We express the Ab concentration as μM precipitated 125I-αBTX binding sites. We measured the concentration of Ab that cross-reacted with mouse MACHR by a modification of the anti-TACHR Ab assay, with Triton X-100 extract of muscles from naive B6 mice as the solubilizing agent of solubilized MACHR, at a concentration of ~1 nM of αBTX binding sites. We labeled the MACHR by overnight incubation with 4 nM 125I-αBTX. We set up precipitation curves by using increasing amounts of serum (0.2–5 μl) in 96-well Immulon-4 Removawell plates (Dynex Technologies, Chantilly, VA), precoated by overnight incubation at 4°C with PBS containing 0.01% sodium azide, 0.05% Tween 20, and 3% BSA (all from Sigma, St. Louis, MO). We used ~0.2 pmol of MACHR per sample, and we usually set up triplicate or quadruplicate samples for each of the serum amounts. After overnight incubation, we precipitated the MACHR-Ab complexes by incubation with 20 μl of Zsorbin (Zymed Laboratories)well for 2 h. All incubations were at 4°C. We washed the plates four times with wash buffer (24) and counted the individual wells in a 5500 gamma counter (Beckman, Fullerton, CA). We express the Ab concentration as nanomolars precipitated 125I-αBTX binding sites.

We used the two-factor ANOVA test with the MacANOVA program to determine the statistical significance between the serum concentration over time of anti-TACHR and anti-MACHR Ab in TG mice and WT mice that had received the same TACHR immunization treatment. We used the following model: response = treatment × time + E(mouse) + treatment × time, where response was the Ab concentration, treatment was the presence (or absence) of the IL-10 transgene, time was the time of the individual test (measured as weeks after the beginning of the immunization), and E(mouse) was the degrees of freedom (a function of the number of the mice in the groups analyzed). We considered a difference to be significant when p ≤ 0.05.

**Assay of serum anti-TACHR IgG subclasses**

We measured by ELISA the anti-TACHR IgG subclasses in sera obtained 4 wk after the second and third TACHR immunizations. We coated ELISA plates (Nunc, Karstrup, Denmark) by overnight incubation at 4°C with 100 μl/well of 25 μg/ml TACHR in 10 mM NaHCO3, pH 9.6. We washed the plates four times with PBS containing 0.05% Tween 20 (PBS-T) and blocked them with 200 μl/well of 2% BSA in PBS-T (PBS-TB) for 1 h at 37°C. After four washings with PBS-T, we added 100 μl/well of three

appropriate serum dilutions (in duplicate) in PBS-TB and incubated the plates overnight at 4°C. We used sera from normal, untreated mice as negative control. We washed the plates six times with PBS-T, added 100 μl/well of 1 μg/ml biotinylated anti-mouse IgG subclass Ab (BD Pharmingen, San Diego, CA) in PBS-TB, and incubated the plates for 1 h at 37°C. We incubated with the IgG2b-labeled streptavidin Abs (1:100 dilutions in PBS of mAbs specific for mouse IgG2b; BD Pharmingen), and AMCA-S-labeled streptavidin (Molecular Probes) diluted, respectively, at 1:4000, 1:100, and 1:200 in PBS containing 3% BSA. We determined the specific absorbance by subtracting the absorbance of the sections three times for 15 min with PBS and viewed them in a fluorescence microscope (Eclipse E800; Nikon, Tokyo, Japan). All procedures were at room temperature. We collected digital images with the program Image Pro Plus (Media Cybernetics, Silver Spring, MD).

Lymphocyte proliferation assay

Five to 7 days after the last TACRH immunization, we obtained splenocytes (24) from two to three identically treated mice. Because we administered the immunizations at multiple spots along the mouse back and conducted the proliferation experiments 5–7 days after the last boost, the spleen is a good source of TACRH-specific CD4+ cells (e.g., Refs. 12, 24, and 26). For the experiments described here, the splenocytes are a better choice than lymph node cells because they can be obtained in large numbers, and this allows both the depletion in CD8+ cells (necessary to assess the cytokine secretion by CD4+ cells), and the recovery of enough cells to test a large number of Ags (necessary to determine the epitope repertoire on the TACRH α subunit). We pooled the splenocytes and depleted them in CD8+ cells using magnetic beads and rat anti-mouse CD8+ Ab (BD Pharmingen). We suspended the CD8+ -depleted splenocytes in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% heat inactivated FCS (Life Technologies), 50 μM 2-ME, 1 mM l-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin (1 × 106 cells/ml), and seeded them in triplicate in 96-well flat-bottomed plates (200 μl/well). We added one of the following Ag or stimulants: 10 μg/ml PHA (Sigma); 5 and 10 μg/ml TACRH, or 10 μg/ml of the individual a subunit peptides. Controls were triplicate wells cultured without any Ag, or with a 20-residue control peptide synthesized by the same method, unrelated to the TACRH sequence (10 μg/ml). After 4 days, we labeled the cells for 16 h with [3H]thymidine (1 μCi per well; sp. act. 6.7 Ci/mmol; DuPont, Boston, MA), harvested them (Titertek, Skatron, Leica, Nublach, Germany). We determined the serum concentration of IgG subclasses in naive WT and TG mice frozen in liquid nitrogen and stored at −80°C. We used tissue from three different WT and TG mice for each group treated with the same Ag for each group (one WT and one TG mice, immunized with 5, 10, or 30 μg of TACRH). We embedded the frozen tissues in OCT Compound Tissue-TEK (Miles Laboratories, Elkhart, IN) and sectioned it in the transverse direction into 10-μm sections with a Jung Frigout 2800E Kryo-stat (Leica, Nublach, Germany). We analyzed at least 10 sections from each mouse.

To detect simultaneously the presence of mouse IgG and complement at muscle, we used hind limb muscle samples of TAChR-immunized or naive WT and TG mice frozen in liquid nitrogen and stored at −80°C. We used tissue from three different WT and TG mice for each group treated with the same Ag for each group (one WT and one TG mice, immunized with 5, 10, or 30 μg of TACRH). We embedded the frozen tissues in OCT Compound Tissue-TEK (Miles Laboratories, Elkhart, IN) and sectioned it in the transverse direction into 10-μm sections with a Jung Frigout 2800E Kryo-stat (Leica, Nublach, Germany). We analyzed at least 10 sections from each mouse.

Detection of IgG, IgG subclasses, and complement at muscle synapses by immunofluorescence microscopy

We used hind limb muscle samples of TACRH-immunized or naive WT and TG mice frozen in liquid nitrogen and stored at −80°C. We used tissue from three different WT and TG mice for each group treated with the same Ag for each group (one WT and one TG mice, immunized with 5, 10, or 30 μg of TACRH). We embedded the frozen tissues in OCT Compound Tissue-TEK (Miles Laboratories, Elkhart, IN) and sectioned it in the transverse direction into 10-μm sections with a Jung Frigout 2800E Kryo-stat (Leica, Nublach, Germany). We analyzed at least 10 sections from each mouse.

To detect simultaneously the presence of mouse IgG and complement at muscle synapses, we selected the sections in PBS for 10 min, and for 1 h with a 1:200 dilution of biotin-conjugated goat anti-mouse IgG polyclonal Ab (Sigma) in PBS containing 3% BSA. We washed the sections with PBS for 15 min three times, and stained them for 1 h with Texas Red-labeled αBTX (Molecular Probes, Eugene, OR), FITC-labeled goat anti-mouse C3 Ab (Nordic Immunological Laboratories, Capistrano Beach, CA), and AMCA-labeled streptavidin (Molecular Probes) diluted, respectively, at 1:4000, 1:100, and 1:200 in PBS containing 3% BSA. For detection of different IgG subclasses, we incubated the sections for 1 h with 1:200 dilutions in PBS of mAbs specific for mouse IgG2a (Sigma; a rat mAb) or IgG2b (BD Pharmingen; mAb anti-mouse IgG2a (Igh-1b) a mouse IgG3 mAb). We washed the sections three times for 15 min with PBS followed by incubation with a biotin-conjugated secondary Ab, which was goat anti-rat IgG polyclonal Ab (Sigma) for IgG2a and a mAb specific for mouse IgG3 (BD Pharmingen) for IgG2b. This was followed by staining with Texas Red-labeled αBTX (Molecular Probes), and ALEXA 350-labeled streptavidin (Molecular Probes), as described above. We washed the sections three times for 15 min with PBS and viewed them in a fluorescence microscopy (Eclipse E800; Nikon, Tokyo, Japan). All procedures were at room temperature. We collected digital images with the program Image Pro Plus (Media Cybernetics, Silver Spring, MD).

Cytokine secretion by CD8+ -depleted spleen cells in response to stimulation with TACRH

Five to 7 days after the last TACRH boost, we prepared CD8+ -depleted splenocytes from two to three identically treated mice. We resuspended the cells at 5 × 106 cells/ml and cultured them with and without 10 μg/ml TACRH in 24-well plates. In some experiments, we set up duplicate cultures. CD8+ -depleted splenocytes cultured without any Ag served as controls for spontaneous secretion of cytokines. We harvested the culture supernatants after 24 and 72 h. We measured the concentrations of IFN-γ, IL-2, IL-4, and IL-10 by capture ELISA, using duplicate samples. We used anti-IFN-γ, anti-IL-2, anti-IL-4, and anti-IL-10 monoclonal and polyclonal Ab (BD Pharmingen), and recombinant IFN-γ, IL-2, IL-4, and IL-10 (BD Pharmingen) as standards, and followed the manufacturer’s instructions.

We determined the significance of the difference in the average cytokine incorporation of [3H]thymidine in cultures exposed to a given Ag and their basal incorporation in the absence of any stimulus by using a two-tailed Students’ t test. We considered a difference to be significant when p < 0.05.

Results

TG mice are very susceptible to EMG

In a first experiment, we immunized 10 WT and 5 TG mice three times with 2 μg of TACRH and used the hanging test to measure their strength every 2–4 wk for 14 wk, starting just before the first TACRH injection (time 0) (Fig. 1A). The shaded area in Fig. 1A (left) represents the holding times (8.3 min or less) that are indicative of EMG. None of the WT mice developed EMG weakness. Some TG mice had reduced holding time at week 4, and most or all of them after week 10: at this time the difference in the average holding time of TG and WT mice was significant (bar in left panel of Fig. 1A). The dashed lines in the left panels of Fig. 1A represent the holding time below which we consider the mice to have moderate and severe EMG (6.75 and 5.2 min, respectively). In the right panels of Fig. 1A, we represent the results of this experiment as the percentage of mice with clinical EMG at the different times. We have indicated the presence of mild, moderate, and severe disease with increasingly dark shaded areas.

In two other experiments, we immunized TG and WT mice three times with 30 μg of TACRH, a dose that induces EMG in WT B6 mice (4). In the first of those experiments, we used 5 TG 5 five WT mice, and in the second we used 10 TG and 6 WT mice. We used the hanging test to measure their strength every 2–4 wk for 14 wk, starting just before the first TACRH injection (time 0). The two
experiments yielded consistent results, which are reported together in Fig. 1B. Similar to Fig. 1A, we report in the left panels the holding times of the individual mice observed in the different tests. They illustrate well the consistency and reliability of the holding test we used. In the right panels, we represent the results of the same experiments as the percentage of mice with clinical EMG at the different times. Among the mice with EMG, we have differentiated, as in Fig. 1A, the presence of mild, moderate, or severe disease. TG mice developed more frequent and more severe EMG than WT mice. About one-third of the WT mice had EMG, usually mild or moderate, during the first 10 wk. At week 12, all WT mice had EMG, which was mild in many of them, but 50% of them had recovered by the end of the observation period. Of the TG mice, 75% had EMG during the first 9 wk, which was moderate or severe in 50% of the animals. By week 11, all TG mice had EMG, which was usually moderate or severe and persisted for the whole duration of the observation period. The difference in the holding times of TG and WT mice was significant for the whole duration of the observation period \( (p < 0.05; \text{horizontal bar in the left panel of Fig. 1B}). \)

**Serum anti-AChR Ab concentrations in TG and WT mice**

We measured by RIPA the anti-TAChR Ab concentration in sera of WT and TG mice immunized with low or high doses of TAChR.

Fig. 2A reports the serum anti-TAChR Ab concentrations of individual mice immunized with 2 \( \mu g \) of TAChR, and their averages. The sera were obtained 4, 8, and 14 wk after beginning the TAChR immunization. In agreement with previous reports (4), we found mouse-to-mouse variations of the serum anti-TAChR Ab. At each tested time, the average Ab concentration in TG mice was higher than in WT mice. The difference between the curves reflecting the time course of the appearance of serum anti-TAChR Ab in the two strains was significantly different \( (p < 0.001). \)

Also after immunization with 30 \( \mu g \) of TAChR, the TG mice developed higher concentrations of serum anti-TAChR Ab than the WT mice. Fig. 2B reports the serum anti-TAChR Ab concentrations of individual mice in the first group immunized with the high TAChR dose and their averages. The sera were obtained 4, 8, and 14 wk after beginning the TAChR immunization. Also in these groups, there was mouse-to-mouse variation in the serum concentration of anti-TAChR Ab. At each tested time, the average anti-TAChR Ab concentration of TG mice was higher than that of the WT mice. The difference between the curves reflecting the time course of the appearance of serum anti-TAChR Ab in the two strains was highly significant \( (p < 0.0015). \)

We measured by RIPA the concentration of Ab that cross-reacted with MACHR in the sera of WT and TG mice immunized with low or high doses of TAChR. Assay of the anti-MACHR Ab, the concentration of which in EMG induced by TAChR immunization is much lower than that of the anti-TAChR Ab (4), required rather large amounts of serum (up to 30–40 \( \mu l/curve \)). For a few mice, we did not have enough serum, at least for some of the time.
experimental details.

high-dose (p) MaChR Ab. By week 14, five of the 13 TG mice tested and six of the mice tested and three of the six WT mice had measurable anti-MaChR Ab, had died. At 11 wk six of the nine surviving TG mice in both the TG and the WT groups had serum anti-MAChR Ab. By this time one TG mouse, which at 6 wk had 8.48 nM serum enough serum for the week 14 samples. Fig. 3 summarizes the wk, at 8 wk, and at 14 wk. For the second experiments, we col-
described above. For both experiments we collected sera at 2– 4
mice immunized with 30 μg of TAChR in the two experiments

points. As controls we used sera of WT mice sham-immunized with adjuvant alone in the same amounts and with the same in-
jection schedule as the TAChR-immunizations.

We measured the serum anti-MAChR Ab concentration of in-
dividual WT and TG mice immunized with 2 μg of TAChR. The sera were obtained 4, 8, and 14 wk after beginning the TAChR immunization as indicated below the plots. In both A and B, the top plots report the Ab concentrations in the sera of individual mice and the bottom plots the average concentrations ± SD in the two groups. At all times TG mice had higher concentrations of anti-TAChR Ab than the WT mice, both after low-dose (p < 0.001) and high-dose (p < 0.0015) immunization. See Materials and Methods for experimental details.

![FIGURE 2](image)

TG mice had more serum anti-TAChR Ab than WT mice. Anti-TAChR Ab concentration, measured by RIPA, in the sera of WT mice (○) and TG mice (●), immunized with injections of 2 μg of TAChR (A) or 30 μg of TAChR (B) at weeks 0, 4, and 8. We obtained the sera 4, 8, and 14 wk after beginning the TAChR immunization as indicated below the plots. In both A and B, the top plots report the Ab concentrations in the sera of individual mice and the bottom plots the average concentrations ± SD in the two groups. At all times TG mice had higher concentrations of anti-TAChR Ab than the WT mice, both after low-dose (p < 0.001) and high-dose (p < 0.0015) immunization. See Materials and Methods for experimental details.

We measured the anti-MAChR Ab in the sera of WT and TG mice immunized with 30 μg of TAChR in the two experiments described above. For both experiments we collected sera at 2–4 wk, at 8 wk, and at 14 wk. For the second experiments, we collected sera also at 6 and 11 wk. For a few mice, we did not have enough serum for the week 14 samples. Fig. 3 summarizes the results of both those experiments.

After the first TAChR injection at 2–4 wk, only two WT mice and one TG mouse had very small amounts of serum anti-MACHR Ab. After 6 wk, the WT mice still did not have anti-MACHR Ab, whereas five of the 10 TG mice tested had anti-MACHR Ab, in high concentrations in two of them (39 and 61 nM) and moderate concentrations in another two (8.5 and 10 nM). At 8 wk, several mice in both the TG and the WT groups had serum-MACHR Ab. By this time one TG mouse, which at 6 wk had 8.48 nM serum anti-MaChR Ab, had died. At 11 wk six of the nine surviving TG mice tested and three of the six WT mice had measurable anti-MaChR Ab. By week 14, five of the 13 TG mice tested and six of the 11 WT mice tested had Ab. The TG mice included the mice with the highest anti-MACHR Ab concentrations. From 6 wk onwards, the average of the serum anti-MACHR Ab concentration of individual mice was higher in the TG than in the WT mice (black symbols in Fig. 3). However, the difference between the two groups was not significant.

Anti-TAChR IgG subclasses in WT and TG mice

We measured the percentage of anti-TAChR IgG subclasses in sera obtained 4, 8, and 14 wk after beginning the TAChR immunization in the mice immunized with low doses of TAChR and in the mice used in the first experiment in which we used high-dose TAChR immunization. In WT mice, the anti-TAChR Ab have been reported to be IgG2b and IgG1, with undetectable IgG3 (15, 17). The presence of IgG2c had not been investigated previously.

Fig. 4A reports the averages of the relative concentrations of anti-TAChR IgG1, IgG2b, and IgG2c in sera of individual mice immunized with the low TAChR dose. We do not show the values for the anti-AChR IgG3 because they were undetectable. WT mice synthesized primarily IgG1 at weeks 4 and 8 and comparable amounts of IgG1 and IgG2b by week 14. At all time points, the IgG2c were a minor component of the anti-TAChR Ab. In contrast, TG mice synthesized primarily anti-TAChR IgG2b at all time points tested. They also synthesized substantial amounts of anti-TAChR IgG2c (~30% of the anti-TAChR Ab at weeks 8 and 14), whereas the synthesis of anti-TAChR IgG 1 was minimal (<10%) at weeks 8 and 14). The relative serum concentration of anti-TAChR IgG1 was significantly lower in the TG mice than in the WT mice at all the time points. The relative concentrations of anti-TAChR IgG2b and IgG2c were significantly higher in the TG mouse sera at all the time points, with the only exception of IgG2c at week 4.

Fig. 4B reports the averages of the relative concentrations of anti-TAChR IgG1, IgG2b, and IgG2c in sera of individual mice immunized with the high TAChR dose. They were the same in TG and WT mice at all the time points, whereas IgG3 were undetectable.

We measured amounts of serum IgG subclasses in naive WT and TG to see whether the two strains have a different profiles in IgG subclass production: no difference was detected (not shown).

**TG and WT mice immunized with TAChR have IgG and complement at the neuromuscular junctions**

We used immunofluorescence to determine the presence of mouse IgG and complement at the neuromuscular junctions of WT and TG mice immunized with either 2 or 30 μg of TAChR. We used
three mice for each group. As negative controls for unspecific binding of the fluorescent probes to muscle, we used muscle sections from three naive WT mice. For each mouse we analyzed at least 10 muscle sections in which we could identify neuromuscular junctions by the binding of fluorescent α-BTX (red fluorescence). We identified the presence of mouse IgG, IgG2b, IgG2c, and the C3 complement component by binding of specific fluorescent Ab (blue and green fluorescence, respectively). Fig. 5 reports representative muscle sections from WT and TG mice immunized with 30 μg of TACHR. All mice of both strains had IgG and complement bound to the neuromuscular junctions, and they had both IgG2b and IgG2c. Also WT and TG mice immunized with 2 μg of TACHR had detectable IgG and complement at the neuromuscular junctions (data not shown). We could not detect IgG, IgG2b, IgG2c or complement at the neuromuscular junctions of naive mice (not shown).

CD4+ T cell response to TACHR and TACHR epitopes in WT and TG mice

After TACHR immunization, CD4+ cells of mice with B6 background recognize primarily epitopes within the sequence region 146–169 of the TACHR α subunit (24, 26, 30–32). CD4+ cells that recognize the immunodominant sequence 146–169 are strongly pathogenic, because lack of recognition of the sequence by CD4+ cells correlates with resistance to EMG induction (30–32). They recognize also epitopes within residues 181–200 and 360–378 of the TACHR α subunit (24) and other minor epitopes (26, 31). We wished to examine whether the increased susceptibility to EMG of the TG mice correlated with enhanced recognition of the TACHR by their CD4+ cells, or with recognition of a different CD4+ epitope repertoire. We used CD8+−depleted splenocytes from WT and TG mice immunized with 30 or 2 μg of TACHR, to determine their proliferative response to the TACHR and to overlapping synthetic peptides spanning the TACHR α subunit sequence.

We conducted one experiment with WT mice and two experiments with TG mice immunized with 30 μg of TACHR that yielded consistent results (Fig. 6). Both strains recognized TACHR vigorously. The CD8+−depleted splenocytes from TG mice recognized the TACHR less strongly than those from the WT mice. Given the limited number of experiments, we cannot decide whether this was attributable to a reduced Th1 response (22) or to the described variations in the proliferative response of individual mice to the TACHR (15, 17, 18, 24, 26, 27). CD8+−depleted splenocytes from WT and TG mice immunized with 30 μg of TACHR recognized a similar repertoire of a subunit peptides. They always recognized most strongly the peptides spanning the immunodominant sequence region 146–169. Other peptides were recognized less consistently and/or less intensely. WT mice recognized also peptides α30−47 and α360−378. TG mice also recognized, at least in one experiment, peptides α1–20, α30−47, α63–80, and α276–295.

CD8+−depleted splenocytes from TG mice immunized three times with 2 μg of TACHR had small yet significant responses to the TACHR, to a pool of the peptides spanning the α subunit sequence, and to the peptides spanning the sequence regions 146–169, 360–378, and 30–47 (data not shown).

Thus, these experiments did not detect any substantial change in the pattern of the recognition of TACHR α subunit epitopes in the TG mice.

Cytokine secretion by anti-TACHR CD4+ cells

We investigated the production of cytokines in response to TACHR stimulation in vitro by CD8+−depleted splenocytes of WT and TG mice immunized with 30 μg of TACHR. We cultured CD8+−depleted splenocytes with TACHR or without any stimulus and measured IFN-γ, IL-2, IL-4, and IL-10 in the culture supernatants by
responses of CD8\(^{+}\) cytocytes secreted more IL-10 and less IFN-\(\gamma\) with splenocytes from WT mice, and two experi-
ments with splenocytes from TG mice. The columns represent average
peptides are indicated at the bottom of the panels. The mice had been
immunized three times with TAChR, at weeks 0, 4, and 8, and had received
a further boost 5–7 days before being sacrificed (at week 15). We con-
ducted one experiment with splenocytes from WT mice, and two experi-
ments with splenocytes from TG mice. The columns represent average
cpm ± SD of triplicate cultures (*, \(p < 0.05\); **, \(p < 0.005\); ***, \(p < 0.0005\)). The baseline values obtained in the absence of any stimulus, or in
the presence of a synthetic 20-residue peptide unrelated to the TAChR
sequence, have been subtracted, and they were: 8602 ± 503 cpm for the
WT (top) and 4511 ± 654 and 5519 ± 898 cpm for the TG mice (middle
and bottom, respectively). See Materials and Methods for experimental
details.

FIGURE 7. Cytokine secretion by CD8\(^{+}\)-depleted splenocytes from
WT and IL-10 TG mice immunized with TAChR after challenge in vitro
with TAChR. IFN-\(\gamma\) and IL-2 (top), IL-4 and IL-10 (bottom) in the super-
natant of cultures of CD8\(^{+}\)-depleted splenocytes from WT mice (□) and
TG mice (■) immunized with TAChR. The mice had been immunized
three times with 30 \(\mu\)g of TAChR at weeks 0, 4, and 8, and had received
a further boost 5–7 days before being sacrificed. The columns represent
the average ± SD of duplicate ELISA determinations with supernatant of cells
cultured in the presence of 10 \(\mu\)g/mI TAChR. The spontaneous secretion of
the different cytokines by cells cultivated without any stimulus was sub-
tracted from these data. The star indicates a significant (\(p < 0.02\)) differ-
ence in the secretion of cytokines by the cells from the TG mice, as com-
pared with the secretion by cells from WT mice (n.s., not significant). See
Materials and Methods for experimental details.

Discussion
We demonstrate here that TG expression of IL-10 simultaneous
with IL-2 synthesis facilitates the synthesis of anti-TAChR Ab and
increases the susceptibility to EMG. At low TAChR doses, only TG mice developed EMG (Fig. 1A). At high TAChR doses, TG mice developed EMG earlier and more frequently than WT mice and their disease persisted for the whole period of observation
(Fig. 1B), whereas in WT mice, several had recovered by the end
of the observation period (Fig. 1B). The higher susceptibility to
EMG of the TG mice was likely attributable to their increased
synthesis of anti-TAChR Ab and of IgG subclasses able to bind
complement and cause damage of the neuromuscular junction
(Figs. 2 and 4).

The TG expression of IL-10 did not have a significant effect on
the serum concentration of anti-MAChR Ab, although after im-
umunization with 30 \(\mu\)g of TAChR, anti-MAChR Ab appeared in the
sera of TG mice earlier than in WT mice, and the few mice with high concentrations of serum anti-MAChR Ab were in the TG
(Fig. 3). Also, after immunization with low doses of TA-
ChR, TG mice had serum anti-MAChR Ab more frequently than
WT mice. The immune cross-reactivity between the TAChR and
MACH is small, and anti-MAChR Ab may accumulate in the
serum only after the MAChR in the mouse muscles has been sat-
urated. Mice have a large MAChR content, and Ab binding to
muscle AChR accelerates its degradation and resynthesis (4), and
therefore absorption of more anti-MAChR Ab. Thus, the absence
or the presence of low amounts of anti-MAChR Ab in the serum
may not correlate with absent or mild EMG symptoms. A lack of
correlation between the concentration of anti-MAChR Ab and se-
verity of EMG in mice has been described (33). Also in MG, there
is poor or no correlation between the concentration of anti-AChR
Ab in the serum and the symptom severity (4). Paradoxically, al-
though Ab cross reactive with MAChR are the direct cause of
EMG, measurement of the total concentration of the anti-TAChR
Ab in our mice provided a better indicator of the effect of TG IL-10
expression on the anti-AChR Ab response.

Complement activation at the neuromuscular junction is a likely
pathogenic mechanism in EMG. For example, IL-12-deficient
mice do not develop EMG after TAChR immunization and com-
plement cannot be detected at their endplates (12). However, despite
the increased synthesis of anti-TAChR IgG subclasses that bind
complement in TG mice immunized with low doses of TAChR, we
could not detect an increased deposition of complement at the neu-
romuscular junctions of TG mice, as compared with WT mice: all
mice immunized with TAChR had C3 at their endplates. The im-
munofluorescence method we used to detect complement is strictly
qualitative and may not detect small yet clinically significant
changes in the amount of complement at the neuromuscular junction.

IL-10 facilitates the differentiation and proliferation of B cells
and production of Ig. It is a switch factor for a variety of Ig classes
and subclasses (34–38) and possibly for all IgG isotypes (39). At
the higher TAChR doses, the percentages of all anti-TAChR IgG
subclasses were similar in WT and TG mice (Fig. 4B), indicating
that TG mice had increased synthesis of all anti-TAChR IgG sub-
classes, including those induced by Th1 cytokines. This suggests
that the increased synthesis of anti-TAChR Ab in TG mice was
caused by the action of IL-10 on B cells, not by the reduced anti-TAChR Th1 response (Fig. 7). This is verified by the finding that immunization of the TG mice with suboptimal doses of TACHR resulted in preferential synthesis of Th1-induced IgG2b and IgG2c (Fig. 4A). This is likely explained by the focal synthesis of the TG IL-10 by Th1 cells simultaneous with IL-2 synthesis. In WT mice immunized with low TACHR doses, the small amounts of anti-TAChR Ab were primarily Th2-induced IgG1 after the first two TACHR injections, and even after the third injection (week 14) the IgG1 were almost half of the anti-TAChR IgG (Fig. 4A). This is consistent with a preferential activation of Th2 cells when their TCR are ligated at low density by the MHC class II/epitope complexes (3, 21).

The action of IL-10 on B cells may be synergistic with IL-2, with or without additional costimulatory signals (40–43). The TG expression of IL-10 in our TG mice might have been especially effective in stimulating IgG synthesis by anti-TAChR B cells, because it occurred simultaneously with secretion of IL-2, and at the same location. The TG mice do not have overall increased levels of serum IgG or of individual IgG subclasses (Ref. 22 and this study), yet they had significantly higher concentrations of anti-TAChR Ab than WT mice (Fig. 2). This argues for a specific stimulatory effect of the TG IL-10 on the B cells that synthesize anti-TAChR Ab, resulting from the cognate interaction between TACHR-specific CD4+ Th and B cells.

The TG expression of IL-10 occurs simultaneously with that of IL-2, which might also contribute to EMG development and to the proliferation and differentiation of anti-TAChR B cells. However, IL-2 is unlikely to have had a role in the enhancement of EMG development in the TG mice, because the IL-2 synthesis induced by challenge with TACHR in cultures of CD8−-depleted splenocytes of TACHR-immunized mice was modestly, albeit not significantly, decreased in the TG mice as compared with the WT mice. This is consistent with the decreased synthesis of IFN-γ (Fig. 7), described previously in TG mice (22), which suggests that secretion of TG IL-10 by the Th1 cells down-regulates the activity of the Th1 cells that express the transgene. An important role of IL-10 in EMG development is supported by studies of IL-10 gene knockout mice, which appeared to be resistant to EMG induction (P. Christadoss, unpublished observations).

IL-10 down-regulates Th1 responses by reducing the expression of costimulatory molecules and cytokines by APC (44–47) and by inhibiting the transcription of the IL-12 genes during the primary Ag stimulus (48). IL-10 also affects CD4+ T cells and resting T cells by inhibiting IL-2 production and T cell growth (49, 50). It induces a long-term Ag-specific anergic state in human CD4+ cells when present during Ag challenge (51, 52) and might be responsible for the increased T cell death mediated by Fas/Fas ligand that occurs in human systemic lupus (53). Also, the shift toward Th2 responses caused by TGF-β may be attributable to inhibition of Th1 cells mediated by IL-10 (54). The decreased secretion of IFN-γ by CD8−-depleted splenocytes from our TG mice, after stimulation in vitro with TACHR (Fig. 7), suggests that they had a reduced Th1 response to the TACHR. This might have occurred because the activated Th1 cells down-regulated their own activity because of the TG IL-10 expression induced by the stimulation of IL-2 synthesis. Some Th1-mediated responses, like anti-tumor activity and colitis, were reduced in the TG mice we used (22).

IL-10, especially in combination with IL-2, facilitates CD8+ CTL responses (55–59). The role of CD8+ cells in EMG is not clear. Mice deficient in MHC class I molecules and CD8+ cells developed EMG with high frequency after immunization with AChR (60), suggesting that CD8+ cells do not have a pathogenic role in EMG. However, other studies suggested that CD8+ cells are necessary for EMG development. In Lewis rats, Ab-mediated depletion of CD8+ cells suppressed EMG development and reduced the synthesis of anti-AChR Ab (61). Also, mutant B6 mice that lacked CD8+ cells did not develop EMG and had reduced anti-AChR Ab responses (62). If CD8+ cells have a facilitating role in rodent EMG, a stimulation of CD8+ cells by the TG IL-10 may have a role in the increased susceptibility of the TG mice to EMG.

Nasal, s.c., or oral administration of TACHR sequences to B6 mice caused activation of specific Th2 cells and protected from EMG (e.g., 15–20). How can these results be reconciled with those we report here? Activated Th2 cells secrete IL-4, which might help prevent EMG directly because of its anti-inflammatory properties and its down-regulating action on Th1 cells (1–3). Also, IL-4 may be a growth factor for modulatory CD4+ cells that secrete TGF-β (also referred to as Th3 cells, Refs. 62–64; however, see Ref. 65). The TGF-β family of cytokines are potent immunomodulators (66) that polarize CD4+ responses toward a Th2 phenotype (66, 67) and block the effects of IL-12 in the development of Th1 responses (66, 68, 69). Because Th3 cells do not produce IL-4, they may be dependent on Th2 cells for proliferative signals (3, 63). Thus, Th2 cells may down-regulate immune responses indirectly, through the action of IL-4-induced Th3 cells.

In WT mice, activation of anti-TACHR Th2 cells might prevent EMG because the protective action of IL-4 overshadows the stimulation of B cells by IL-10. IL-10 itself may down-regulate Th1 cells and APC, and contribute to protect from EMG after s.c. or nasal “tolerization” procedures. TG mice did not have an increased production of IL-4 (Fig. 7). Moreover, their transient secretion of large amounts of IL-10 by activated anti-TACHR Th1 cells that also secreted IL-2 enhanced the anti-TACHR B cell response. In TG mice, the anti-TACHR Th1 cells were down-regulated by their own TG IL-10 secretion, because they produced less IFN-γ than WT mice. However, this effect did not suffice to curb the overstimulation of the anti-TACHR Ab response.

Because of its ability to down-regulate Th1 cells and the synthesis of a variety of cytokines, IL-10 is considered as a possible therapy of undesirable immune responses (70, 71). However, the effects of IL-10 on T cell-mediated experimental autoimmune responses are complex and conflicting. For example, in nonobese diabetic mice, TG pancreatic expression of IL-10 accelerated autoimmune diabetes, and treatment with anti-IL-10 Ab prevented insulitis (72–74). Yet treatment with IL-10 or an adoptive transfer of islet specific T clones transfected with IL-10 cDNA prevented diabetes (75–77). The regulated production of IL-10 in T cells by the transgene we used here had a weak inhibitory effect in some mouse models of diabetes (23). Also in experimental autoimmune encephalomyelitis (EAE) the effects of IL-10 are contrasting. IL-10 TG mice were resistant to EAE, and genetically IL-10-deficient mice were more susceptible than WT mice (78). Yet administration of IL-10 did not affect EAE development (79). IL-10 has an important pathogenic role in an Ab-mediated autoimmune disease, systemic lupus erythematosus (SLE). In SLE, the Ig production is IL-10 dependent, and an increased production of IL-10 by B cells and monocytes may be a critical pathogenic mechanism (80, 81). The finding that MG patients have increased blood levels of AChR-specific cells that secrete IL-10 is consistent with a pathogenic role of this cytokine in MG (82).

These results, and the pathogenic role of IL-10 in SLE (80, 81), raise concerns about the suitability of IL-10 to curb Ab-mediated autoimmune diseases, and suggest that IL-10 might be a target, rather than a tool, in the suppression of undesirable Ab responses.
References


