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Absence of IFN-γ or IL-12 Has Different Effects on Experimental Myasthenia Gravis in C57BL/6 Mice

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Immunization with acetylcholine receptor (AChR) causes experimental myasthenia gravis (EMG). Th1 cells facilitate EMG development. IFN-γ and IL-12 induce Th1 responses: we investigated whether these cytokines are necessary for EMG development. We immunized wild-type (WT) C57BL/6 mice and IFN-γ and IL-12 knockout mutants (IFN-γ−/−, IL-12−/−) with Torpedo AChR (TACHR). WT and IFN-γ−/− mice developed EMG with similar frequency, IL-12−/− mice were resistant to EMG. All strains synthesized anti-AChR Ab that were not IgM or IgE. WT mice had anti-AChR IgG1, IgG2b, and IgG2c, IFN-γ−/− mice had significantly less IgG2c, and IL-12−/− mice less IgG2b and IgG2c. All mice had IgG bound to muscle synapses, but only WT and IFN-γ−/− mice had complement; WT mice had both IgG2b and IgG2c, IFN-γ−/− only IgG2b, and IL-12−/− neither IgG2b nor IgG2c. CD4+ cells from all AChR-immunized mice proliferated in response to AChR and recognized similar epitopes. After stimulation with TACHR, CD4+ cells from IFN-γ−/− mice secreted less IL-2 and similar amounts of IL-4 and IL-10 as WT mice. CD4+ cells from IFN-γ−/− mice secreted less IFN-γ, but more IL-4 and IL-10 than WT mice, suggesting that they developed a stronger Th2 response to TACHR. The EMG resistance of IL-12−/− mice is likely due to both reduction of anti-TACHR Ab that bind complement and sensitization of modulatory Th2 cells. The reduced Th1 function of IFN-γ−/− mice does not suffice to reduce all complement-fixing IgG subclasses, perhaps because as in WT a protective Th2 response is missing.

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D4+ cells comprise Th1 and Th2 cells that differ in their function and the cytokines they secrete (1–4). Th1 cells mediate effector functions of the immune response. They secrete proinflammatory cytokines, such as IFN-γ and IL-2, can be cytotoxic, and help the synthesis of IgG subclasses that bind complement. Th1 cells have been implicated in the pathogenesis of Ab-mediated autoimmune diseases, because they help synthesis of Ab able to fix complement, that should be especially effective in causing tissue damage (1–4). IL-12 and IFN-γ are potent inducers of Th1 cell differentiation (1, 4). IL-12 facilitates Th1 responses by stimulating the differentiation of naive Th cells into Th1 cells, and by serving as a costimulus for maximum IFN-γ secretion by Ag-activated Th1 cells (5–6). Activated Th1 cells secrete IFN-γ, which promotes further Th1 cell proliferation and activates macrophages (1, 4). IFN-γ-deficient mice have reduced Th1 and macrophage function (7). Mice genetically deficient in IL-12 have severely reduced Th1 responses and increased secretion of IL-4 after Ag stimulation, suggesting a polarization of their immune responses toward Th2 sensitization (8).

Myasthenia gravis (MG)4 and its animal model, experimental MG (EMG), are caused by Ab to the muscle acetylcholine receptor (AChR) (9). Th1 cells are involved in the pathogenesis of MG and EMG. MG patients had AChR-specific Th1 cells (10–13). IL-4 knockout mice, that have a reduced Th2 function but a normal Th1 function, were susceptible to EMG after immunization with AChR (14–15). IL-12 knockout mice (IL-12−/−) immunized with AChR synthesized anti-AChR Ab, but they seldom developed electromyographical abnormalities indicative of reduced neuromuscular transmission that were frequent in AChR-immunized wild-type (WT) mice (16). Administration of IL-12 at the time of the AChR immunization facilitated the appearance of abnormality of the neuromuscular transmission revealed by electromyography (16). Mice deficient in IFN-γ and immunized with AChR did not develop anti-AChR Ab or EMG symptoms (17), whereas mice genetically deficient in IFN-γ receptor developed anti-AChR Ab, although in less amounts than WT mice, and presented EMG, but less frequently than WT mice (18). Those studies (17, 18) suggest that IFN-γ affects EMG development by complex mechanisms that are different from those influenced by IL-12. This is supported by the finding that transgenic mice that produced IFN-γ at the neuromuscular junction did not develop anti-AChR Ab, yet they had functional disruption of the junction and clinical weakness reminiscent of MG (19).

Th2 cells help synthesis of Ab that do not bind complement, such as IgA, IgE, and IgG1 in mice (1–4), and are unlikely to cause severe tissue injury. They modulate immune responses by secreting anti-inflammatory cytokines, like IL-4 and IL-10, that down-regulate the function of APC and Th1 cells (1–4). Also, Th2 cells may down-regulate immune responses indirectly by stimulating the activity of modulatory CD4+ cells that secrete TGF-β (sometimes referred to as Th3 cells (4)). The TGF-β family of cytokines are potent immunomodulators (20) that can polarize CD4+ responses toward a Th2 phenotype (21, 22) and block the effects of IL-12 in the development of Th1 responses (23, 24).

Experimental Myasthenia Gravis in C57BL/6 Mice

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Abbreviations used in this paper: MG, myasthenia gravis; α-BTX, α-bungarotoxin; AChR, muscle acetylcholine receptor; EMG, experimental MG; NMS, normal mouse serum; TACHR, Torpedo AChR; RIPA, radioimmunoprecipitation assay; WT, wild type; EAE, experimental autoimmune encephalomyelitis.

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cells are dependent on IL-4 for growth, but they do not produce it; therefore, they may be dependent upon Th2 cells for proliferative signals (4, 25, 26).

Th2 and Th3 cells may have a protective role in EMG. Nasal or s.c. administration to C57BL/6 (B6) mice of synthetic AChR peptides forming CD4+ epitopes activated peptide-specific Th2 cells, caused reduced synthesis of anti-AChR Ab, and prevented EMG (27–29). IL-4 knockout B6 mice, that have defective Th2 (and presumably Th3) responses, were more susceptible to EMG than WT B6 mice and were not protected from EMG by nasal administration of synthetic AChR CD4+ epitopes (15). An EMG-resistant rat strain had increased up-regulation of TGF-β as compared with an EMG-susceptible strain (30). Oral administration of purified AChR provided protection from EMG and up-regulated the synthesis of TGF-β by AChR-specific CD4+ cells (31).

IL-12 is the most important cytokine for Th1 cell differentiation, and IFN-γ is an important Th1 effector molecule as well as an autocrine growth factor for Th1 cells (1–4). Here, we investigated the relative importance of these cytokines in the development of EMG. We immunized WT B6 mice, and their IFN-γ and IL-12 knockout (IFN-γ−/−, IL-12−/−) mutants with purified AChR. We determined the appearance of EMG and of anti-AChR Ab, their Ig isotypes and IgG subclasses in sera, and the presence of IgG, IgG subclasses, and complement at the neuromuscular junction. Also, we assessed the proliferative response of the CD4+ cells from WT, IFN-γ−/−, and IL-12−/− mice to the TACChR, their epitope repertoire, and the cytokines they secreted after challenge in vitro with AChR.

Materials and Methods

Mice

We bought WT, IFN-γ−/−, and IL-12−/− B6 mice from The Jackson Laboratory (Bar Harbor, ME) and bred them at the animal facility of the University of Minnesota. Because of the unexpected sensitivity to EMG found for the IFN-γ−/− mice (see below), we verified the genotypes of 10 randomly selected IFN-γ−/− and 5 WT mice by PCR using DNA from 3 to 5 μl of blood from each mouse, prepared using Chelex 100 resin (Bio-Rad, Hercules, CA) (32). One microliter of supernatant of the Chelex extract was used as a template for PCR. Each reaction mixture (total volume, 10 μl) included 1 μl 10× PCR buffer II (Perkin-Elmer, Foster City, CA), 1 μl of 10× dNTPs (10× dNTPs: 2 mM each dATP, dCTP, dGTP, and dUTP) (Pharmacia, Piscataway, NJ)), 0.5 μl of 25 mM MgCl2 solution (Perkin-Elmer), 1 μl of 10 μM stock solutions for each of the primers of the pairs listed below, 2 μl of red sucrose PCR-compatible loading dye (60% sucrose and 1 mM cresol red), 0.1 μl of 5 U/μl Ampli Taq Gold DNA polymerase (Perkin-Elmer), and 3.1 μl of sterile double-distilled H2O. Each sample was covered with 30 μl of mineral oil. For PCR, the samples were hot started for 10 min at 95°C, followed by a three-step cycling profile using 35 cycles of 30 s at 95°C, 30 s at 53°C, and 45 s at 72°C in a Perkin-Elmer model 9600 DNA thermal cycler. We used the following primers, as recommended by The Jackson Laboratory: for the WT IFN-γ allele, IMR126−5′-AGA AGT AAG TGG AAG GGC CCA GAA G-3′ and IMR127−5′-AGG GAA ACT GGG AGA GGA GAA ATA T-3′ (the resulting product is 220 bp); and for the disrupted IFN-γ allele, IMR128−5′-TCA GCC GAG GGG CGC CCG GTT CTT T-3′ and IMR129−5′-ATC GAC AAG AAC GCC TTC CAT CCG A-3′ (the resulting product is 375 bp). We used both pairs of primers for each mouse DNA: each pair was used in a separate tube, so that only one product could be amplified for each reaction. We assessed the size of the products by 1.5% agarose (Life Technologies, Gaithersburg, MD) gel electrophoresis, stained with 0.001% ethidium bromide (Sigma, St. Louis, MO). When we used DNA from WT mice, we obtained a PCR product of 220 bp only when we used the primers corresponding to the unmutated IFN-γ sequence. When we used DNA from IFN-γ−/− mice, we obtained a PCR product of 375 bp only when we used the primers corresponding to the mutated IFN-γ sequence.

Purification of Torpedo AChR (TACChR)

We purified AChR from the electric tissue of Torpedo californica fish as alkali-stripped AChR-rich membrane fragments (TACChR)3 (33). The AChR structure is highly conserved along evolution: TACChR is quite similar to mammalian muscle AChR and suitable to induce EMG (9). We measured the protein concentration by the Lowry assay (34), the AChR protein content as α-bungarotoxin (α-BTX) binding sites (33), and we assessed the protein composition by SDS-PAGE (35). The TACChR preparations we used contained 3.8–5.8 nmol of sites/mg protein and contained only the four TACChR subunits as the main protein bands. For use in cell cultures, we diluted the TACChR-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 (33), diluted them to 0.5 mg/ml in PBS, and stored them at −80°C.

Pepitides

To determine the epitope repertoire on the TACChR α subunit, which dominates the sensitization of anti-TACChR CD4+ cells in B6 mice (33), we used a panel of synthetic (36) peptides, ~20-residue long and overlapping by ~5 residues that spanned the sequence of the TACChR α subunit. We reported their characterization previously (33). We used solutions of the individual peptides in PBS sterilized by UV irradiation and stored frozen.

TACChR immunization

We immunized 8- to 10 wk-old female mice by s.c. injections, along the border of the tail of the base of the tail, with solubilized TAChR (20–40 μg in 100 μl PBS) emulsified in an equal volume of CFA. We boosted them twice at 4-wk intervals with the same amount of TACChR in incomplete Freund’s adjuvant and a third time 5–7 days before being euthanized at the end of week 15.

Evaluation of clinical symptoms of EMG

We quantified the EMG symptoms using an inverted hang-forced exercise, sensitized by a minute amount of pancuronium bromide (0.03 mg/kg i.p.), given just before the test (37). The mice hang from a grid: we measured the time it took for the mouse to release its hold and fall three times (“holding time”). The test is parametric and gives a quantitative assessment of the severity of the weakness of the mouse. To verify the myasthenic nature of the weakness, we injected edrophonium chloride (Reversol; Organon, West Orange, NJ) intraperitoneal. Reversol is a cholinesterase inhibitor which immediately increased the strength of mice that have EMG. We tested the mice every 1–3 wk, starting on the day of the first immunization. We performed the test without knowledge of the treatment that the mouse had received.

The average holding time of 285 naive WT mice was 11.4 ± 1.55 min. Naïve IL-12−/− and IFN-γ−/− mice had holding times indistinguishable from WT mice. We considered myasthenic mice with holding times of 8.3 min (the holding time of normal mice minus 2 SD) or less. Paralyzed mice or mice that died of respiratory paralysis are represented in the figures as having holding time of zero.

Anti-AChR Ab assay

We obtained sera after each clinical testing. We measured the serum concentration of anti-TACChR Ab by radioimmunoprecipitation assay (RIPA) using TACChR solubilized in Triton X-100 and labeled by the binding of 125I-α-BTX (38). This assay uses as precipitating Ab a polyclonal serum raised against mouse Ig that include the α and λ light chains shared by all Ig; therefore, the serum precipitates all Ig isotypes and IgG subclasses. We express the Ab concentration as micromolar precipitated 125I-α-BTX binding sites.

Assay of anti-TACChR IgM, IgE, IgG, and IgG subclasses

We measured by ELISA the anti-TACChR IgM, IgE, IgG, and the IgG subclasses in mouse sera obtained at 8 and 14 wk after the first TACChR immunization. We coated ELISA plates (Nunc, Karstrup, Denmark) by overnight incubation at 4°C with 100 μl/well of 25 μg/ml TACChR 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

3 We use TACChR to indicate the AChR purified from Torpedo electric organ, whereas AChR indicates AChR irrespective of its tissue source and animal species. In this paper, the abbreviation AChR frequently refers to mouse muscle AChR.
duplicate) in PBS-T and incubated the plates overnight at 4°C. We used serum from normal untreated mice (NMS) as a negative control. We washed the plates six times with PBS-T, added 100 μl/well of 1 μg/ml biotinylated IgM, IgG, total IgG, or anti-mouse Ab (PharMingen, San Diego, CA) in PBS-T, and incubated the plates for 1 h at 37°C. Mice carrying the IgG-1 allele, like B6 mice, do not express IgG2a, but rather a related isotype, termed IgG2b or IgG2c (39, 40). To detect anti-TAChR IgG2c in the mouse sera, we used the mAb dion anti-mouse anti-IgG2b (IgG-1) (PharMingen).

After eight washings with PBS-T, we added 100 μl/well of 1 μg/ml avidin-peroxidase (Sigma, St. Louis, MO) and the plates for 1 h at 37°C. We washed the plates eight times with PBS-T and added 100 μl/well of 0.3 mg/ml 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) in 0.01% H2O2/citric acid buffer (ABTS peroxidase substrate system; Kirkegaard & Perry Laboratories, Gaithersburg, MD). After development of the blue-green color at room temperature, we stopped the enzymatic reaction by adding 100 μl/well of 1% SDS. We measured the absorbance at 405 nm with an automated microplate reader ELx800 (Bio-Tek Instruments, Winooski, VT). We determined the specific absorbance by subtracting the absorbance of wells incubated with NMS dilutions from that of wells incubated with the sera from TACaR-immunized mice. The serum concentration of anti-TAChR isotypes and IgG subclasses was inferred from standard curves obtained by coating ELISA plates directly with 100 μl/well of purified murine total IgG (Sigma), IgM, and IgE (PharMingen) or IgG1, IgG2a, and IgG2b subclasses (Sigma) from 100 to 0.007 μg/ml in 10 mM NaHCO3 (pH 9.6) and detecting their presence with the biotinylated anti-mouse Ig class or Ig subclass/avidin-peroxidase system, as described above. An ideal standard curve would have required the use of purified anti-AChR Ig subclasses binding to the TACaR coating the plate. Therefore, the values of Ig and IgG subclass concentrations in serum we report here should not be considered as absolute concentrations. However, since our goal was to assess the relative amounts of the different anti-AChR Ig classes and IgG subclasses synthesized in IFN-γ- or IL-12-/- mice as compared with WT mice, we considered our standard curve a suitable reference for our evaluations, because it represents an internal standard that allows comparisons of results for the individual isotypes and subclasses obtained in different experiments. We could not construct a standard curve for IgG2c because purified IgG2c is not commercially available. Thus, for IgG2c we simply report the values of OD, expressed as OD units at a wavelength of 405 nm, obtained for sera of the three mouse strains assayed simultaneously. We obtained the results reported here doing duplicate determinations of three serum dilutions (1:30,000, 1:90,000, and 1:270,000), the OD values in the linear range were normalized as to represent an arbitrary dilution of 1:90,000 and were averaged.

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

Hind limb muscle tissue of WT, IFN-γ-/-, and IL-12-/- mice, frozen in liquid nitrogen and stored at −70°C, was embedded in OCT Compound Tissue-Tek (Miles Laboratories, Elkhart, IN) and sectioned it in the transverse direction into 10-μm sections using a Jung Frigout 2800E Kryostat (Leica, Nublach, Germany). To detect simultaneously the presence of mouse IgG and complement at the neuromuscular junction, we incubated the sections at room temperature for 10 min in PBS and for 1 h with a 1:200 dilution of rabbit anti-goat 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 at room temperature with Texas Red-labeled α-BTX (Molecular Probes, Eugene, OR), FITC-labeled goat anti-mouse C3 Ab (Nordic Immunological Laboratories, Capistrano Beach, CA), and 6-(7-amino-4-methylcoumarin)-3-(2-methylimidazolyl)coumarin (Molecular Probes) diluted in PBS containing 3% BSA at 1:4000, 1:100, and 1:200 dilutions, respectively. For detection of mouse IgM and different IgG subclasses, we incubated the sections for 1 h with 1:20 dilutions in PBS of goat anti-mouse IgM Ab (Sigma) or mAbs specific for mouse IgG1 (a rat mAb; Sigma), IgG2a (a rat mAb; Sigma), and IgG2c (PharMingen) Ab anti-mouse IgG2b (IgG-1); a mouse IgG3 mAb. The sections were washed with PBS for 15 min three times, followed by incubation with a biotin-conjugated secondary Ab, which was Texas Red-labeled goat IgG polyclonal Ab (Sigma) for IgM, goat anti-rat IgG polyclonal Ab (Sigma) for IgG1 and IgG2b, and a mAb specific for mouse IgG3 (PharMingen) for IgG2c. This was followed by staining with Texas Red-labeled α-BTX (Molecular Probes), FITC-labeled goat anti-mouse C3 Ab (Nordic Immunological Laboratories), and ALEXA 540-labeled streptavidin (Molecular Probes) as described above. We washed the sections three times with PBS and viewed them in a fluorescent microscope (Nikon Eclipse E800; Nikon Diaphot, Melville, NY). We collected digital images using the program Image Pro Plus (Media Cybernetics, Silver Spring, MD).

Assay of Ab bound to mouse muscle AChR

We measured the Ab bound to mouse muscle AChR by RIPA (33) as follows. Carcasses from three to five mice were skinned, eviscerated, and homogenized individually in two volumes of 50 mM Tris-HCl, 50 mM NaCl, 5 mM EDTA, 20 mM PMSF, and 1.2 mM EGTA (pH 7.4, extraction buffer). The homogenates were centrifuged at 30,000 rpm for 60 min. The cell pellets obtained were resuspended in extraction buffer and extracted with 2% Triton X-100 for 4 h under shaking. We centrifuged the extract at 30,000 rpm for 60 min and collected the supernatant. We added 4 pmol of 1125-α-BTX to each quadruplicate of 1-ml aliquots of Triton X-100 extract of each mouse carcass and incubated them for 24 h. Five microliters of NMS was added to each aliquot. Each hundred microliters per sample anti-mouse IgG Ab were added. The samples were incubated overnight, centrifuged at 12,000 rpm, washed four times with 10 mM PBS (pH 7.4) containing 0.1% Triton X-100, and counted in a gamma 5500 counter (Beckman, Irvine, CA). All procedures were conducted at 4°C.

Proliferation assay

Five to 7 days after the last immunization, we obtained spleen cells (33) from two to three identically treated mice and pooled them. This was necessary to obtain enough cells for testing all of the Ag listed below. We depleted the splenocytes in CD8+ and displayed magnetic beads and rat anti-mouse CD8 Ab (PharMingen) (33, 37, 38).

For proliferation assays, we suspended the cells in RPMI 1640 (Life Technologies) 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). We seeded the cells in triplicate in 96-well flat-bottom plates (200 μl/well). We added one of the following Ag or stimulants: 10 μg/ml PHA (Sigma), 10 μg/ml TACaR, and 10 μg/ml of the individual α subunit peptides. Controls were triplicate wells cultivated without any Ag or with a 20-residue control peptide synthesized by the same method, unrelated to the TACaR sequence (10 μg/ml). After 4 days, we labeled the cells for 16 h with [3H]thymidine (1 μCi/well, sp. act., 6.7 Ci/mmol; DuPont, Boston, MA) and harvested the cells (Titertek, Skatron, Sterling, VA). We measured the [3H]thymidine incorporation by liquid scintillation.

Cytokine secretion by CD8+-depleted splenocytes in response to stimulation with TACaR

Five to 7 days after a last boost with TACaR, we prepared pooled CD8+-depleted splenocytes from three identically treated mice. We resuspended the cells at 5 × 10⁶ cells/ml and cultured them with and without 10 μg/ml TACaR in 24-well plates. In some experiments, we set up two independent cultures for each Ag. Cells were cultivated with an equal volume of extraction buffer and with spontaneous secretion of cytokines. We harvested the culture supernatants after 24 and 96 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 (PharMingen) and recombinant IFN-γ, IL-2, IL-4, and IL-10 (PharMingen) as standards according to the manufacturer’s instructions.

Statistical analysis

We determined the significance of the differences of the average responses of two groups using a two-tailed Student’s t test. We considered a difference significant when p < 0.05. To determine the significance of the difference between two curves, such as those describing the time course of the appearance of anti-AChR Ab, we used the program EXCEL-ANOVA. We considered a difference to be significant when p < 0.05.

Results

IL-12-deficient mice are resistant to EMG, whereas IFN-γ-deficient mice are susceptible

In the first set of experiments, we immunized five WT, five IFN-γ-/- and four IL-12-/- mice with TACaR. We measured the strength of the individual mice every 2 wk for 14 wk, starting just before the first TACaR injection (time 0, Fig. 1). The horizontal line in Fig. 1 corresponds to the holding time (8.3 min) below which we considered the mice to have EMG. Two WT mice developed persistent myasthenic weakness from week 10 onward. The other three mice had holding times indicative of EMG between 11 and 12 wk and recovered by week 14 (i.e., 6 wk after the third TACaR immunization). None of the IL-12-/- mice developed stable EMG weakness, although two mice had a transient
reduction of the holding time to values slightly below 8.3 min, indicating a modest reduction of the function of the neuromuscular transmission. All IFN-γ−/− mice developed EMG. Three had abnormally reduced holding time after the second TACHR injection when the WT mice still had normal holding times. From week 9 onward, four IFN-γ−/− mice had stable EMG and one had a transient reduction of the holding time to values slightly below 8.3 min, but recovered by week 14.

To verify the susceptibility of IFN-γ−/− mice to EMG, we immunized a second set of mice that included eight IFN-γ−/− mice and 19 WT mice. We tested the mouse strength just before the first TACHR immunization and every 2 wk for 10 wk. The results of the tests conducted 4, 8, and 10 wk after the beginning of the immunization are reported in Fig. 2. IFN-γ−/− and WT mice developed EMG with similar frequency and severity.

WT, IFN-γ−/−, and IL-12γ−/− mice develop comparable amounts of anti-TACHR Ab

We measured the serum anti-TACHR Ab of WT, IFN-γ−/−, and IL-12γ−/− mice using the RIPA. Fig. 3 shows the anti-TACHR Ab concentration in the sera of the mice shown in Fig. 1 that was obtained 4, 8, and 14 wk after the beginning of the TACHR immunization. In agreement with previous reports (9), we found substantial variations in the concentration of anti-TACHR Ab from mouse to mouse that are reflected in the large SDs of the values in Fig. 3. The average concentration of serum anti-TACHR Ab was similar for all three groups. We obtained similar results when we used the sera of the IFN-γ−/− and WT mice illustrated in Fig. 2. After three immunizations with TACHR, WT and IFN-γ−/− mice had average serum concentrations of anti-TACHR Ab of 2.8 ± 1.1 and 2.1 ± 0.7 μM: the difference was not statistically significant.

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FIGURE 1. IFN-γ−/− mice are susceptible to EMG, whereas IL-12γ−/− mice are resistant. Strength of five WT, five IFN-γ−/−, and four IL-12γ−/−, as indicated inside the panels, measured by the pancuronium-sensitized hanging test at the times indicated. The mice received three TACHR injections at the times indicated with arrows (0, 4, and 8 wk). The tests at 0, 4, and 8 wk were conducted just before the TACHR-immunizing injections. The horizontal lines indicate a holding time of 8.3 min (holding time of normal mice minus 2 SD). Mice with a holding time of 8.3 min or less were considered to have EMG. See text for experimental details.

FIGURE 2. IFN-γ−/− mice are as susceptible to EMG as WT mice. Strength of IFN-γ−/− and WT mice, as indicated at the right side of the panels, measured by the pancuronium-sensitized hanging test. The tests were conducted for 4, 8, and 10 wk after beginning the immunization as indicated below the panels. The mice had received three TACHR injections (at weeks 0, 4, and 8). The horizontal lines indicate a holding time of 8.3 min. See text for experimental details.

FIGURE 3. IFN-γ−/− and IL-12γ−/− mutants had similar concentrations of anti-TACHR Ab as WT mice. Average anti-TACHR Ab concentration (±SD) measured by RIPA in the sera of five WT mice, five IFN-γ−/−, and four IL-12γ−/− mutants immunized with TACHR. We obtained the sera at 4, 8, and 14 weeks after the beginning of the TACHR immunization, as indicated along the abscissa. The mice had received three TACHR injections (at weeks 0, 4, and 8). See text for experimental details.

FIGURE 4. Concentration of different anti-TACHR IgG subclasses in the sera of WT, IFN-γ−/−, and IL-12γ−/− mice. The mice had received three TACHR injections (at weeks 0, 4, and 8). The sera were obtained 4, 8, and 14 wk after beginning of the TACHR immunization. In agreement with previous reports (9), we found substantial variations in the concentration of anti-TACHR Ab from mouse to mouse that are reflected in the large SDs of the values in Fig. 3. The average concentration of serum anti-TACHR Ab was similar for all three groups. We obtained similar results when we used the sera of the IFN-γ−/− and WT mice illustrated in Fig. 2. After three immunizations with TACHR, WT and IFN-γ−/− mice had average serum concentrations of anti-TACHR Ab of 2.8 ± 1.1 and 2.1 ± 0.7 μM: the difference was not statistically significant.
Differential effects of IFN-γ and IL-12 deficiency on the synthesis of Th1-driven IgG subclasses

We measured by ELISA the serum concentration of anti-TAChR IgG subclasses driven by different cytokines. WT mice synthesized primarily anti-TAChR IgG2b and IgG2c, to a lesser extent IgG1 (Fig. 4), while IgG3 was undetectable (data not shown); the anti-IgG2a mAb yielded a very small signal that likely resulted from cross-reactivity with the highly homologous IgG2c (Fig. 4). IFN-γ−/− and IL-12−/− mice had anti-TAChR IgG1 in amounts similar to those of the WT mice, whereas their anti-TAChR IgG2c (and IgG2a) was strongly reduced: in both strains, they were almost undetectable even 14 wk after beginning the TACChR immunization (Fig. 4). Absence of IFN-γ or IL-12 had different effects on synthesis of anti-TAChR IgG2b. They were significantly reduced in IL-12−/− mice, whereas in IFN-γ−/− mice their serum concentration was comparable to that of WT mice. IFN-γ−/− mice had a slight reduction in anti-TAChR IgG2b at week 14, which was not significant as compared with WT mice.

The data reported in Fig. 4 suggest that the overall serum concentration of anti-TAChR IgG was higher in WT mice than in the cytokine mutants. On the other hand, the RIPA experiments indicated that all strains had similar amounts of serum anti-TAChR Ab. To identify reasons for this discrepancy, we determined by ELISA the concentration of anti-TAChR IgG, IgM, and IgE in the mouse sera. They all had large amounts of anti-TAChR IgG, although IFN-γ−/− and IL-12−/− mice had less IgG than WT mice: after three TACChR injections, the average total IgG in IFN-γ−/− and IL-12−/− mice was 50 and 40%, respectively, of the average total IgG in WT mice. None of the strains had measurable serum anti-TAChR IgM or IgE.

IFN-γ−/− and WT mice immunized with TACChR have IgG2b and complement at the neuromuscular junction, whereas IL-12−/− mice do not

We investigated whether the different susceptibility to EMG of these strains was related to the ability of the anti-TAChR Ab to bind to muscle and fix complement.

We measured by RIPA the amounts of mouse muscle AChR that was bound to Ab in three WT mice, four IFN-γ−/− mice, and three IL-12−/− mice immunized three times with TACChR and boosted with a fourth injection 5–7 days before euthanasia. We found comparable amounts of Ab-complexed AChR in all three groups (Fig. 5A). Thus, the EMG resistance of the IL-12−/− mice was not due to inability of their anti-TAChR Ab to bind muscle AChR.

We used immunofluorescence to determine the presence of mouse IgG and complement at the neuromuscular junctions of three WT, three IFN-γ−/−, and three IL-12−/− mice immunized with TACChR. For each mouse, we analyzed at least 10 muscle sections in which we could identify neuromuscular junctions by α-BTX binding (red fluorescence). We identified mouse IgG and the C3 complement component by specific fluorescent Ab (blue and green fluorescence, respectively). Fig. 5B shows the results of representative sections for each strain. All mice had IgG bound to the neuromuscular junctions. All WT and IFN-γ−/− mice also had complement, whereas we could not detect the presence of complement in any muscle section from the IL-12−/− mice.

We investigated whether the presence of complement at the neuromuscular junctions correlated with binding of complement-fixing IgG subclasses. We stained muscle sections of a mouse from each strain with α-BTX and with mAbs specific for IgG2b and IgG2c. Other sections of the same mice were stained for total IgG, 

![Figure 5](http://www.jimmunol.org/Downloadedfrom/Experimentalyastheniainfernt-gelylandil-deficiencymice.png)

**FIGURE 5.** A. Concentration of muscle AChR complexed by Ab in the muscle of mice that had been immunized four times with TACChR as described above (at weeks 0, 4, 8, and 14), measured by RIPA. The mice had been sacrificed 5–7 days after the last TACChR immunization. See text for experimental details. B. Presence of mouse IgG and complement at the neuromuscular junction of IFN-γ−/− and IL-12−/− and WT mice. Muscle sections from mice of the different strains, as indicated at the right, were immunized with TACChR as described above and sacrificed 5–7 days after the last TACChR immunization. The sections were stained for mouse IgG (blue fluorescence) and the C3 component of complement (green fluorescence) as indicated below the panels. We localized the AChR at the neuromuscular synapses using triple immunofluorescence staining with binding of α-BTX (red fluorescence) as indicated below the panels. Original magnification, ×1000. See text for experimental details.
IgM, and complement. All mice had IgG and none had IgM bound to the junctions; only WT and IFN-γ mice had complement. When stained for the different IgG2 subclasses, the neuromuscular junctions of the WT mouse stained for both IgG2b and IgG2c, those of the IFN-γ−/− mouse stained only for IgG2b, whereas those of the IL-12−/− mouse did not stain for either IgG2b or IgG2c (Fig. 5C).

CD4+ T cell response to TACR and TACR epitopes in WT, IFN-γ−/−, and IL-12−/− mice

CD4+ cells of mice of B6 background immunized with TACR recognize primarily an epitope or overlapping epitopes within the sequence region 146–169 of the TACR α subunit (35, 37, 38, 41–42), and less intensely and consistently epitopes within the sequences 181–200 and 360–378 of the TACR α subunit (33) and other minor epitopes (38). We used CD8+−depleted splenocytes from WT, IFN-γ−/−, and IL-12−/− mice immunized three times with TACR and boosted with another TACR injection 5–7 days before being euthanized to determine their proliferative response to the TACR and to overlapping synthetic peptides spanning the sequence of the TACR α subunit. We conducted two independent experiments that yielded consistent results. Fig. 6 illustrates the results of one experiment. All strains recognized the TACR vigorously and to comparable extents. They all recognized the peptides spanning the sequence region 146–169 and peptide 360–378. Other peptides were recognized inconsistently. In the experiment depicted in Fig. 6, WT mice also recognized the sequence α30–47, IFN-γ−/− mice also recognized peptides α106–122 and α181–200, and IL-12−/− mice also recognized the peptides spanning the sequence α30–60 and α420–438.

Cytokine secretion by anti-TACR CD8−−depleted spleen cells

Stimulation of modulatory Th2 or Th3 cells, which depend upon IL-4 for their development, is a possible mechanism of protection from EMG. We investigated whether an increased anti-TACR sensitization of Th2 cells correlated with resistance to EMG by measuring the IL-4 and IL-10 secreted by CD8−−depleted spleen cells challenged in vitro with TACR. We determined also the secretion of IFN-γ and IL-2 in the same cultures. We used CD8−−depleted splenocytes from mice immunized three times with TACR and boosted with another TACR injection 5–7 days before being euthanized to determine their proliferative response to the TACR and to overlapping synthetic peptides spanning the sequence of the TACR α subunit. We conducted two independent experiments that yielded consistent results. Fig. 6 illustrates the results of one experiment. All strains recognized the TACR vigorously and to comparable extents. They all recognized the peptides spanning the sequence region 146–169 and peptide 360–378. Other peptides were recognized inconsistently. In the experiment depicted in Fig. 6, WT mice also recognized the sequence α30–47, IFN-γ−/− mice also recognized peptides α106–122 and α181–200, and IL-12−/− mice also recognized the peptides spanning the sequence α30–60 and α420–438.

Discussion

We demonstrate here that in B6 mice IL-12 and IFN-γ have different roles in the pathogenesis of an autoantibody-mediated disease, EMG. IL-12, and therefore Th1 cells, have a crucial role, since IL-12−/− mice had minimal or no EMG symptoms: this is in excellent agreement with the conclusions of a previous study (16). On the other hand, in B6 mice IFN-γ is not necessary for EMG development, since IFN-γ−/− mice developed anti-TACR Ab (Fig. 3) and EMG symptoms (Figs. 1 and 2) with the same frequency and intensity as WT mice.

Two studies (17, 18) investigated the effects of disrupting the genes for IFN-γ and its receptor on EMG using mice of mixed genetic background ((129/SvEv × C57BL/6)F2). The 129/SvEv and the C57BL/6 strains both have the H-2b allele, and the I-A allele influences EMG susceptibility; however, genetic factors unrelated to the MHC haplotype also influence susceptibility to EMG (43–45). The different genetic background of the mice used in those studies (17, 18) and in the present study complicates the comparison of their results.

One study (17) found that (129/SvEv × C57BL/6)F2 mice genetically deficient in IFN-γ did not synthesize anti-TACR Ab (including Th2-driven IgG subclasses) or develop EMG after TACR immunization. Absence of IFN-γ reduces the effectiveness of Ag presentation. IFN-γ−/− mice have reduced macrophage function and expression of MHC class II molecules (7). An insufficient immunization, perhaps due to TACR degradation by proteases that copurify with the TACR (46–47), might explain the lack of anti-TACR response in that study (17).

The second study (18) found that (129/SvEv × C57BL/6)F2 mice genetically deficient in IFN-γ receptor developed less anti-AChR Ab than the nonmutated mice and less frequent and severe EMG. This indicated that IFN-γ signaling was a disposable, albeit facilitating, factor for EMG development. Thus, EMG can develop in the absence of IFN-γ signaling if the TACR immunization suffices to elicit a good anti-AChR Ab response.

The more important facilitating effect of IFN-γ on EMG in (129/SvEv × C57BL/6)F2 (17, 18) than in B6 mice may be because it stimulates macrophage function and expression of class II molecules (1–4). Also, IFN-γ may trigger pathogenic mechanisms other than those mediated by anti-TACR Ab: transgenic expression of IFN-γ in mouse muscle resulted in cellular infiltrates, deposition of IgG at the neuromuscular junction, and disruption of neuromuscular transmission without synthesis of anti-AChR Ab (19). IFN-γ has complex and sometimes contrasting effects in other autoimmune responses (see below). This and the previous studies (17, 18) indicate that also in EMG the effects of IFN-γ are complex and may be influenced by other genetic factors.

The serum concentration of anti-AChR Ab does not correlate with symptom severity in EMG or MG, indicating that not every subpopulation of anti-AChR Ab causes the disease (9). In agreement with those findings, the mouse strains we used, irrespective of their susceptibility to EMG, had levels of serum anti-TACR Ab that were not statistically different. Since none of the strains developed anti-TACR IgM or IgE and the cytokine mutants had less anti-TACR IgG, the similar levels of anti-TACR Ab found in the RIPA experiments in all strains might be due to the presence of anti-TACR IgA in the Th1 cytokine-deficient mice, because of their propensity to develop Th2-induced Ab responses (7, 8). In our mice the ability of anti-TACR Ab to induce EMG was not due just to cross-reactivity with muscle AChR, since all IL-12−/− mice had Ab bound to their neuromuscular junctions (Fig. 5A), yet they did not have EMG weakness. The ability of anti-AChR Ab to fix complement likely contributes to their pathogenic potential.

The diminished ability to synthesize complement-binding anti-TACR IgG subclasses in EMG-resistant IL-12−/− mice (Fig. 4) and the absence of complement and of complement-binding IgG2b and IgG2c at their muscle synapses (Fig. 5, B and C) support an important role of complement activation in EMG development.

WT mice made large amounts of anti-TACR IgG2b and 2e (Fig. 4), which bind complement effectively. IFN-γ−/− mice had less anti-TACR IgG2c, whereas the IL-12−/− had less of both anti-TACR IgG2b and IgG2c (Fig. 4). Also, IL-12−/− mice was the only strain that did not have IgG2b (and complement) at the muscle synapses. These data suggest that anti-AChR IgG2b have
and they were: for the WT mice, 8602 obtained in the absence of any stimulus or in the presence of a synthetic TAChR Th2 cells. This is suggested by the increased secretion of protective mechanism might be the increased sensitization of anti-AChR IgG2c in EMG. Theserum concentration of anti-TAChR reducd synthesis of anti-TAChR IgG2c (Fig. 4), yet were susceptible to EMG (Figs. 1 and 2) down plays the importance of the absence of any stimulus was subtracted. The asterisks indicate a significant difference in the secretion of cytokines by the cells of the knockout mutants as compared with the secretion by the cells from WT mice (*, p < 0.03; **, p < 0.02; ***, p < 0.01, ****, p < 0.002). See text for experimental details.

a prominent role in triggering the complement-mediated destruction of the neuromuscular junction. The effective synthesis of anti-TAChR IgG2b and their binding to mouse muscle AChR likely explain the consistent presence of complement at the neuromuscular junctions of IFN-γ−/− mice (Fig. 5) and their frequent EMG symptoms (Figs. 1 and 2). The finding that IFN-γ−/− mice had a reduced synthesis of anti-TAChR IgG2c (Fig. 4), yet were susceptible to EMG (Figs. 1 and 2) down plays the importance of the anti-AChR IgG2c in EMG. The serum concentration of anti-TAChR IgG1 was comparable in all strains, suggesting that Th2-driven IgG subclasses have limited importance in EMG development.

Two IL-12−/− mice after three TACRHr immunizations had reduced holding times indicative of EMG (Fig. 1). Also Moioli et al. (16) found that a few IL-12−/− mice immunized with TACRHr had defective neuromuscular transmission. This might be due to the small amounts of anti-TAChR IgG2b in these mice, perhaps driven by the action of IL-2 or other cytokines (48).

Other mechanisms might cause the reduced synthesis of pathogenic anti-AChR Ab and the EMG resistance of IL-12−/− mice. A protective mechanism might be the increased sensitization of anti-TAChR Th2 cells. This is suggested by the increased secretion of IL-4 and IL-10 after challenge with TACRHr by splenocytes from IL-12−/− mice as compared with WT and IFN-γ−/− mice (Fig. 7). Also Moioli et al. (16) found that IL-12−/− B6 mice had increased TACRHr-specific IL-4 producing cells. Anti-TACRHr Th2 cells might have a direct protective action by down-regulating the activity of Th1 cells and APC and by exerting a local anti-inflammatory activity. They may also exert indirect protective effects mediated by IL-4 through the activation of regulatory cells that secrete TGF-β (4, 49).

All three mice strains we tested had a good sensitization of CD4+ cells to the TACRHr. Their spleen CD4+ cells recognized most strongly the same sequence regions of the TACRHr α subunit (residues 146–169 and residues 360–378, Fig. 6). We observed small differences in the CD4+ repertoire of WT- and cytokine-deficient mice, perhaps due to individual variations in the CD4+ response to minor TACRHr epitopes (9). One exception may be peptide 420–438, that elicited a strong response of CD4+ splenocytes from IL-12−/− mice: CD4+ cells from WT- or IL-4-deficient B6 mice never recognized this sequence (Refs. 15, 33, 37, 38, 41, and 42 and Fig. 6), that might sensitize Th2 cells.

Development of several T cell-mediated experimental autoimmune diseases requires IL-12 (50–55). IL-12 has been implicated also in the pathogenesis of multiple sclerosis (56, 57). In those diseases, Th1 effector cells cause tissue injury by local secretion of proinflammatory cytokines (50–59). Development of Ab-mediated autoimmune diseases does not always require IL-12 (60–62).

IFN-γ has complex and contrasting effects in the development of T cell-mediated autoimmune diseases. Most EAE-inducing T cell clones isolated from diseased mice secreted IFN-γ (62) and Ab that neutralized IFN-γ-inducing factor prevented EAE (59). Treatment with IFN-γ exacerbated multiple sclerosis (63). Yet, mice with a disrupted IFN-γ gene were susceptible to EAE (64), and IFN-γ may even play a role in down-regulating EAE (65). IL-12, but not IFN-γ, appeared to be crucial for development of mouse Th1 cell-mediated colitis (66, 67). IFN-γ transgenic rats that expressed IFN-γ in the eye had increased severity and accelerated onset of autoimmune uveitis (68), yet IFN-γ-deficient mice could develop this disease (69). Transgenic expression of IFN-γ in pancreatic β cells caused loss of islet tolerance (58), and treatment of nonobese diabetic mice with anti-IFN-γ Ab prevented diabetes (70–71); yet, genetic absence of IFN-γ in nonobese diabetic mice delayed but did not prevent diabetes (72).

The present results underline the importance of IL-12 and complement-induced destruction of the neuromuscular junction in the development of EMG. Complement-fixing Ab are likely important in MG as well, since MG patients always have complement at the neuromuscular junction (9) and anti-AChR Th1 cells (12, 13). The present results suggest that IL-12 might be a target for therapeutic approaches in MG.
References


